THE EFFECTS OF PLANT AND ENVIRONMENTAL FACTORS ON THE NITRATE REDUCTASE ACTIVITY OF BARLEY, *HORDEUM VULGARE*

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ABSTRACT

The nitrate reductase assay *in vivo*, and the effects of plant and environmental factors on winter barley nitrate reductase activity were investigated. The work provides a basis for gauging the potential of this biochemical test as an alternative to largely unsuccessful soil and plant tissue tests for predicting field crop nitrogen status and nitrogen fertiliser top-dressing requirements.

Nitrate reductase (NR) is the substrate inducible plant enzyme which catalyses the reduction of NO_3^- to NO_2^- and is the first, and rate-limiting, step in the biochemical pathway along which NO_3^- is converted into plant protein.

This work describes the development of an assay *in vivo* for NR involving the incubation of fresh barley shoot, or root, material in darkness in a solution containing buffer, solvent and surfactant. Investigations were carried out to determine the optimum incubation medium composition, pH, temperature and plant material preparation conditions. A large number of other pre-, during and post-incubation factors which influence NO_2^- production were also investigated. The object of the optimisation procedure was to maximise NO_2^- accumulation during assays and, hence, to develop a rapid, sensitive and reproducible assay method for use in studies of effects of plant and environmental factors on barley NRA.

Plant factors investigated included the effects of variety, leaf age, leaf position on the plant and the part of the leaf sampled on barley NRA. Environmental factors studied included effects on NRA *in vivo* of light conditions (intensity, time after start of photoperiod, darkness), nutrient pH, root and shoot temperatures and,

especially, nitrogen nutrition. In the studies of effects of nitrogen nutrition on NRA, factors investigated included rates of induction of NRA by NO_3^-, rates of decline in NRA on withdrawal of NO_3^- nutrition, influence of NH_4^+ and concentration of NO_3^- supplied.

In many of the above studies, both induced NRA (NRA_i) and endogenous NRA (NRA_e) values were measured in incubation media with and without added NO_3^- respectively. Ratios of NRA_i to NRA_e were calculated to give nitrate assimilation capacity (NAC) values, which more accurately indicated plant N status than either absolute NRA_e or NRA_i values. NAC values were largely invariant to fluctuations in environmental factors, unlike absolute NRA values which were greatly influenced by many of the plant, environmental and nutritional factors investigated.

Finally, a preliminary field study was made of NRA and NAC in a commercially grown winter barley crop. Results were encouraging enough to warrant the proposal of future field studies to extend the use of NRA and NAC measurements from an indicator of crop N status to a commercially useful predictor of optimum rate and time of application of fertiliser N top-dressing.

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LIST OF ABBREVIATIONS

ADP	:	adenosine diphosphate
ATP	:	adenosine triphosphate
DM	:	dry matter
Fd_{red}	:	reduced ferredoxin
Fd_{ox}	:	oxidised ferredoxin
fr.wt.	:	fresh weight
GDH	:	glutamate dehydrogenase
GOGAT	:	glutamate synthase
GOT	:	glutamate oxaloacetate transaminase
GS	:	glutamine synthetase
MDH	:	malate dehydrogenase
NAC	:	nitrate assimilation capacity
NADH	:	reduced nicotinamide adenine dinucleotide
NED	:	N-(1-naphthyl) ethylene diamine
NiR	:	nitrite reductase enzyme
NiRA	:	nitrite reductase activity
NR	:	nitrate reductase enzyme
NRA	:	nitrate reductase activity
NRA_e	:	endogenous nitrate reductase activity
NRA_i	:	induced nitrate reductase activity
O.D.	:	oven dry
PDSA	:	phenoldisulphonic acid

1. Introduction

Nitrogen is the soil nutrient most commonly required in greatest amounts by crops (Lee & Stewart, 1978), and large quantities of N fertiliser are often applied to supplement soil reserves. The amount of N fertiliser used in 1977, on a global basis, was approximately 43 million tonnes (Beevers & Hageman, 1980). In the United Kingdom alone, the input of N fertiliser to agriculture in 1980 was about 1.3 million tonnes (Royal Society, 1983).

A Royal Society study group recently emphasised the need for further research to improve the efficiency of fertiliser N utilisation and to minimize losses of fertiliser N (Royal Society, 1983). It is important and desirable to optimize both rates and timing of addition of fertiliser N to crops, since supra-optimal applications are not fully utilised and can be easily lost from the soil (mostly in the NO_3^- form) by leaching into drainage water and by denitrification. Denitrification is caused by dissimilatory bacteria in the soil which bring about the reduction of NO_3^- and NO_2^- to the gases NO, N_2O and N_2 which are lost from the soil. These losses of fertiliser N can range from 5-50% of the total applied, especially in wet, poorly aerated soils (Mengel & Kirkby, 1982).

As well as constituting an economic loss to farmers, N unrecovered by the crop represents an energy waste and can cause eutrophication in water courses and pollution of ground water by leaching. In turn, water drawn from polluted aquifers for public consumption may cause health problems, since it has been suggested that NO_3^- can be converted to carcinogenic nitrosamines by human intestinal bacteria (Royal Society, 1983). In addition, drinking water polluted with NO_3^- may also cause methaemoglobinaemia in infants (Royal

Society, 1983). Applications of sub-optimal quantities of fertiliser N can result in serious reductions in potential crop yields and profitability (Royal Society, 1983).

At present, methods of estimating optimum quantities of fertiliser N required by crops are speculative, result in inefficient fertiliser utilisation and, hence, are unsatisfactory. Methods of predicting N application rates include both soil and plant tests and methods based on computer/mathematical modelling of soil/plant systems (Addiscott, 1982 and Greenwood, 1982). Soil tests can be used to predict both basal and top-dressing fertiliser rates. Plant tests, by contrast, are only of use for predicting amounts of N for top-dressing, since basal N applications are made before plants are available for sampling.

There are no simple, rapid soil tests presently available which will effectively estimate amounts of available N present in soils at the start of the growing season. Many of the soil tests tried in the past have involved shaking soil samples with extractants and determining amounts of NH_4^+, NO_3^- or total N released. Extractants used have included acid and alkaline permanganate (Bangar, 1978 and Stanford, 1978), 0.01M $CaCl_2$ or H_2SO_4 (Fox, 1978), saturated $Ca(OH)_2$ solution (James, 1971) and $Ba(OH)_2$ (Jenkinson, 1968). A novel, rapid test has been developed using the ultra-violet absorbance of a 0.01M $NaHCO_3$ extract (Fox, 1978 and Whitehead et al., 1981).

The major drawback of all these tests is that they do not provide information on how much additional inorganic N will be made available later in the growing season by microbial decomposition of soil organic matter i.e. mineralisation. Since rates of mineralisation depend greatly on prevailing weather conditions (Jungk & Wehrmann, 1978), soil tests are of limited use in predicting N fertiliser requirements

of crops. However, soil extracts made with boiling 1M KCl have shown more promise because they include NH_4^+ released from readily hydrolysed soil organic matter in addition to endogenous mineral N. (Oien, 1980; Whitehead, 1981 and Sylvester-Bradley, 1984).

Further information on the amount of potentially available soil N can be obtained from tests based on aerobic or anaerobic incubations of soil samples under controlled conditions. However, these tests can take up to three weeks to perform and can not take account of unexpected variations in weather conditions in the field (Jenkinson, 1968; Jungk & Wehrmann, 1978 and Gomah, 1981).

Methods of predicting fertiliser N requirements by plant analysis have been based on chemical analysis for total N, NO_3^--N or other nitrogenous fractions (e.g. amides, amino acids) to estimate plant N status (Jungk & Wehrmann, 1978). However, total N measurements are unsuitable as indicators of plant N status (Jungk & Wehrmann, 1978 and Verstraeten & Vlassak, 1981) since high total N may be detected in N-deficient plants as a relic effect of a previous, adequate N supply.

The problem with using NO_3^--N levels in dry matter, or petiolar sap as an index of N status is that NO_3^- is present in two seperate pools in plant cells: very small, metabolically active, cytoplasmic pools and potentially much larger, vacuolar storage pools. Excess NO_3^- may be stored in vacuolar pools in times of adequate NO_3^- nutrition and, if plants are analysed at a later date, severe overestimations of N status may result because of stored NO_3^- (Jungk & Wehrmann, 1978 and Verstraeten & Vlassak, 1981).

In addition to difficulties in predicting optimum application rates of fertiliser N, there is also the problem of determining the most appropriate time of application to maximise its utilisation by

crops and minimize N losses by denitrification or leaching. Methods for predicting optimum times to apply fertiliser N include techniques based on plant growth stages (Verstraeten, 1982) and cumulative average air temperatures (T_{sum} methods) (Sylvester-Bradley, 1984).

There exists another, elegant approach which may be of use in predicting both optimum rates and timings of additions of N fertiliser top-dressings. The technique is a form of plant analysis employing living tissues. The method involves measuring the activity of the plant enzyme nitrate reductase (NR) (Jaworski, 1971). NR catalyzes the reduction of NO_3^- to NO_2^-, the first, and rate-limiting, step in the biochemical pathway along which NO_3^- passes as it is metabolized into plant proteins essential for growth (Jones and Sheard, 1978 and Beevers & Hageman, 1980).

Since enzymes are a direct product of a plant's genes, their activities may serve as direct indicators of the plant's metabolic status and potential (Croy & Hageman, 1970 and Kraljevic-Balalic, 1983). It has been proposed that levels of NR activity (NRA) might indicate the rate at which reduced N is being made available for plant growth (Lee & Stewart, 1978 and Beevers & Hageman, 1980). Also, NRA determinations may discriminate between metabolically active, cytoplasmic NO_3^- and inactive vacuolar NO_3^- (Bar-Akiva & Sternbaum, 1965 and Bar-Akiva, 1970). In addition, NRA measurements have been shown to be more sensitive indicators of variations in plant N status than are determinations of total N or petiole NO_3^- concentrations (Bar-Akiva, 1967 and Oosterhuis & Bate, 1983).

A further refinement of the NRA measurement technique involves determining the enzyme's endogenous activity, NRA_e (based on NO_3^- initially present in the plant) and its induced activity, NRA_i (determined with exogenously supplied NO_3^-). The ratio of induced : endogenous

activity ($^{NRA}i/NRA_e$) is usually termed the nitrate assimilation capacity (NAC) and is a better indicator of nitrogen status than endogenous NRA alone, since NAC is less affected by variations in environmental conditions (Bar-Akiva & Sternbaum, 1965 and Bar-Akiva, 1970).

It has been proposed that NRA measurements could be of use in all field circumstances, even when crops are supplied with NH_4^+-based fertilisers, since applied NH_4^+ is rapidly oxidised to NO_3^- by nitrifying bacteria in well-aerated, moist soils at temperatures above 7^oC (Beevers & Hageman, 1980). Also, NO_3^- is the predominant form of soil N likely to be present in well-aerated soils in temperate climates, since NH_4^+ produced by mineralisation of organic matter is rapidly oxidised to NO_2^- by the autotrophic bacteria Nitrosomonas and this NO_2^- is oxidised to NO_3^- by Nitrobacter (Lee & Stewart, 1978). In addition, NO_3^- appears to be the predominant form of N taken up by soil-grown crops (Srivastava, 1980 and Thomson, 1985).

Most workers who have studied NR have only investigated one or two very detailed aspects of its properties e.g. effects of a particular chemical, plant factor or environmental condition on NRA. In contrast, a few workers have made general studies on the properties and potential uses of NR but, unfortunately, most studies have been rather superficial. As a result, both groups of workers have, on the whole, failed to appreciate the potential of NRA measurements as predictors of plant N status, with the notable exceptions of Bar-Akiva and Verstraeten and co-workers.

The overall aim of the work described here therefore, was to initiate a comprehensive study into the use of NR activity in general, and NAC in particular, as indicators of plant N status. Winter barley

was chosen as the test plant species since most of its fertiliser N is applied as a top-dressing in the spring and because current methods of predicting application rates - based speculatively on a knowledge of soil type/previous cropping/weather - provide relatively crude estimates of its optimum N fertiliser requirements (Jungk & Wehrman, 1978). Farmers often apply considerably more or less N fertiliser than necessary which may result in a waste of resources (energy/money), and cause pollution. All these undesirable consequences of applying non-optimal amounts of fertiliser N will be avoided if better estimates can be made of the nitrogen requirements of crops.

The specific objectives of the work reported in this thesis were:

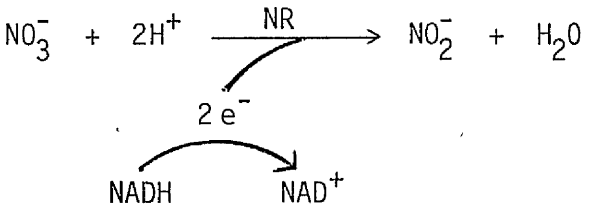
1) to optimise existing semi-quantitative NR measurement techniques to produce a sensitive, reproducible method for determining NRA in barley plants and,
2) to use the assay in vivo technique so developed to study the effects of plant factors and environmental conditions on NRA of winter barley plants grown under controlled conditions.

2. Literature Review

2.1 NO_3^- incorporation into plant protein

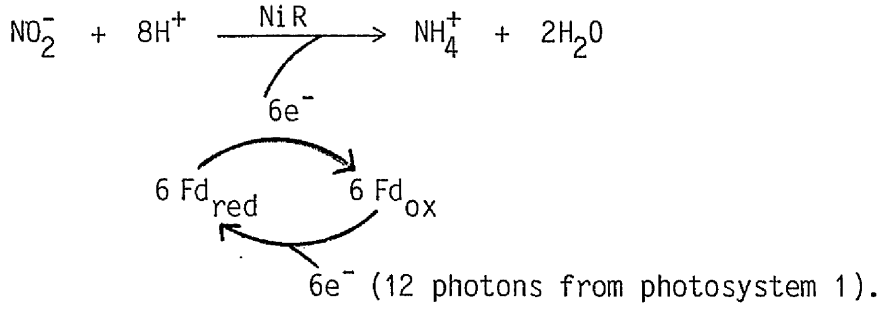
NO_3^- taken up from a growing medium by plants is incorporated into plant protein via several stages. The first of these is the reduction, in the cytoplasm, of NO_3^- to NO_2^- catalysed by the enzyme nitrate reductase, NR (Reaction A).

REACTION A



NO_2^- is then reduced to NH_4^+, the reaction being catalysed by the enzyme nitrite reductase, NiR (EC 1.7.7.1 nitrite oxidoreductase), Reaction B.

REACTION B



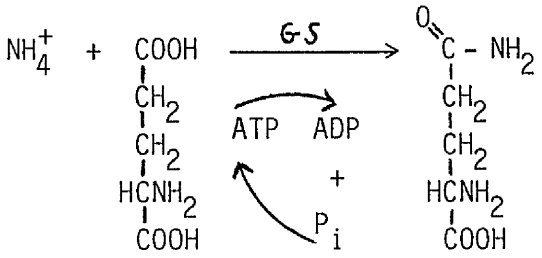
Electrons for this reduction are produced during oxidation of reduced ferredoxin, Fd_{red}.

The overall reaction for the reduction of NO_3^- to NH_4^+ is therefore:

(Guerrero et al., 1981 and Bourne & Miflin, 1973).

NH_4^+ thus produced then reacts with L-glutamate to produce L-glutamine in a reaction catalysed by the enzyme glutamine synthetase (GS), Reaction C.

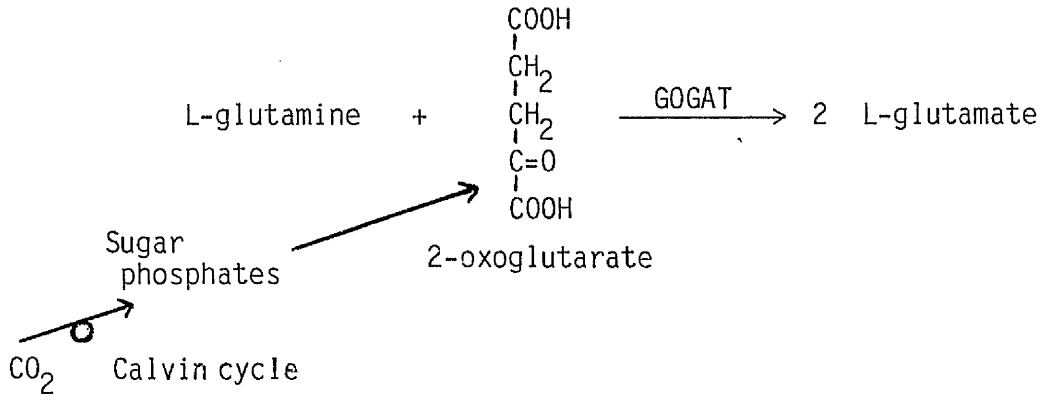
REACTION C



L-glutamate L-glutamine

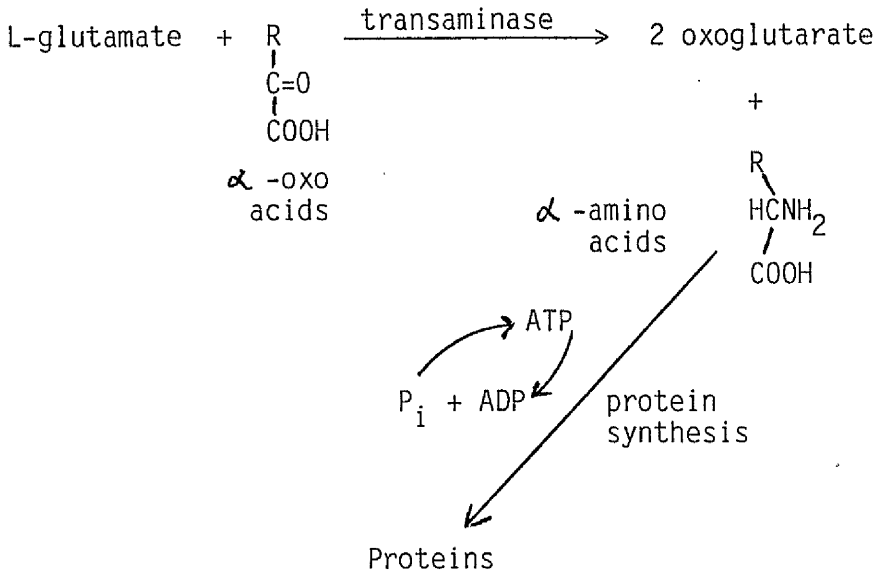
Next, L-glutamine combines with 2-oxoglutarate to produce L-glutamate, the reaction being catalysed by the enzyme glutamate synthase (GOGAT, glutamine 2-oxoglutarate aminotransferase) Reaction D.

REACTION D



Finally, L-glutamate reacts with α-oxo acids to produce α-amino acids which link to form plant proteins, reaction E.

REACTION E



(Miflin & Lea, 1976 and Lee & Stewart, 1978).

2.2 Nitrate reductase

Nitrate reductase (NR, nitrate oxidoreductase EC 1.6.6.1) catalyses the reduction of NO_3^- to NO_2^- in the first stage of the incorporation of NO_3^- into plant proteins, (Section 2.1). NR is one of the relatively few examples of a substrate inducible enzyme in higher plants (Lèe & Stewart, 1978; Beevers & Hageman, 1980).

NR is a metalloflavoprotein and contains Mo, FAD and haeme as prosthetic groups (Nicholas & Nason, 1955 and Wray, 1982). The enzyme has not been extensively purified, but structural studies have indicated that its molecular weight is approximately 280 k Daltons, that it consists of three similar-sized sub-units and that it has a Stokes radius of about 9nm. Functional NR has been proposed to be a dimer of the unit described above (Howard, 1980).

NR has been shown to be located in the cytoplasm (Finke, 1977) and an association with external chloroplast membranes has been

reported (Beevers & Hageman, 1972). In contrast, NiR has been found to be localized within chloroplasts (Finke, 1977).

The usual reductant for NR in plants is NADH (Kadam et al., 1980 and Adam, 1982) derived from photosynthetically produced sugar phosphates by glycolytic activity in the cytoplasm (Beevers & Hageman, 1972). The reductant for NiR is reduced ferredoxin produced in chloroplasts (Finke, 1977).

NR is extremely labile. Its turnover rate is very high in vivo (half life, $t_{\frac{1}{2}} \simeq 4$ hours) (Sluiters-Scholten, 1975) and it is one of the first enzymes to be inactivated by elevated temperatures, thus preventing accumulation of toxic quantities of NO_2^- or NH_4^+ in chloroplasts (Magalhaes et al., 1976).

Nitrate reduction appears to be the rate-limiting step in the conversion of NO_3^- to amino acids and proteins (Verstraeten & Vlassak, 1981), and so only NO_3^- accumulates in plants; while NO_2^- and NH_4^+ do not (Srivastava, 1980). NR therefore effectively regulates nitrogen assimilation in plants (Beevers & Hageman, 1969).

The level of NRA is a function of relative rates of synthesis (or activation) and of decay (or inactivation) (Beevers & Hageman, 1972). Light and substrate (NO_3^-) promote synthesis/activation of NR, while products of nitrate metabolism (NH_4^+, amino acids) inhibit/repress induction and cause decay/inactivation (Beevers & Hageman, 1969).

2.3 Assays for NR

NR can be assayed in vivo or in vitro (Brunetti & Hageman, 1976). Assays in vivo involve incubating fresh plant material in the dark in a buffered solution (Jaworski, 1971). Endogenous NO_3^- in the

material is reduced by the action of NR and the NO_2^- produced diffuses into the incubation medium (an effect initially observed by Kumada (1953)). The rate of NO_2^- accumulation is then taken as a measure of endogenous activity (NRA_e) (Sylvester-Bradley, 1984). If an exogenous NO_3^- supply is added during incubations, a higher, induced activity (NRA_i) may be detected since NR is a substrate-inducible (adaptive) enzyme (Bar-Akiva et al., 1970 and Chopra, 1983).

Assays in vitro involve macerating plant material (Hewitt & Nicholas, 1964) in an extraction medium containing protectants (Streeter & Bosler, 1972) to prevent inactivation or degradation of NR (Lewis et al., 1982). Protectants include cystine and dithiothreitol to protect sulphydryl groups on NR from oxidation (Beevers & Hageman, 1980) and polyvinyl-pyrrolidine and ion-exchange resins to prevent endogenous phenolics from causing protein precipitation (Hageman & Hucklesby, 1971 and Purvis, 1984).

Homogenates resulting from maceration are filtered and centrifuged to provide active NR extracts which are incubated with exogenously supplied substrate (NO_3^-) and reductant (Kaiser & Lewis, 1984). Reductants used in vitro include NAD(P)H, NADH, flavin and benzyl viologen (Hewitt & Nicholas, 1964). During incubation, NO_3^- is reduced to NO_2^- by NR and the rate of NO_2^- accumulation is a measure of NRA.

Both in assays in vivo and in vitro, NO_2^- is usually determined colorimetrically using the Greiss-Ilosvay method (Black, 1965).

2.4 Optimisation of NR assays in vivo

Assay conditions required to maximise NO_2^- production during NR assays in vivo have often been determined to produce sensitive,

reproducible assay techniques (Sylvester-Bradley, 1984). However, optimum conditions have been shown to be dependent on plant species, variety and organ tested (Jones & Sheard, 1977; Perez & Kliewer, 1978; Lin & Kao, 1980 and Sylvester-Bradley, 1984).

2.4.1 Main factors affecting NO_2^- accumulation

a) Solvents and surfactants

Additions of low molecular weight organic solvents to incubation media during NR assays *in vivo* of soyabeans, triticale and beans cause increases in NO_2^- production (Jaworski, 1971; Lin & Kao, 1980 and Puranik & Srivastava, 1983), as do additions of non-ionic surfactants (wetting agents) (Lawrence & Herrick, 1982). The solvent, and its concentration, required to maximise NO_2^- production has been shown to be species-dependent (Jones & Sheard, 1977), while addition of both solvent and surfactant was noted to cause further increases in NO_2^- production during NR assays of maize (Jones & Sheard, 1977).

b) NO_3^-

Addition of NO_3^- to NR assays *in vivo* induces a higher NRA (Hageman *et al.*, 1980) since substrate (NO_3^-), and not reductant (NADH), limits NO_3^- reduction (Nicholas *et al.*, 1976 and Hageman *et al.*, 1980). The amount of exogenous NO_3^- required for maximum NRA is plant species and tissue-dependent (Hageman & Hucklesby, 1971 and Sylvester-Bradley, 1984) in the range 0.01-0.3 M NO_3^-, (Jaworski, 1971; Perez & Kliewer, 1978 and Lin & Kao, 1980). Also, NO_3^- concentrations of greater than 0.5 M were found to inhibit or repress NR of wheat and barley (Sylvester-Bradley, 1984).

c) Buffer

Phosphate has been reported to be superior to TRIS or HEPES buffers in the incubation medium during NR assays of tomato (Sanderson & Cocking, 1964), soybean (Tingey et al., 1974) and wheat and barley tissues (Sylvester-Bradley, 1984). Also, addition of phosphate to NR extracts made with TRIS buffer stimulates NRA (Hageman & Hucklesby, 1971). However, phosphate buffer strength has little effect on NRA as long as buffer concentrations are adequate to prevent organic acids released from plant tissues from lowering the incubation pH (Jaworski, 1971; Jones & Sheard, 1977 and Lin & Kao, 1980).

d) Incubation pH

Although intracellular pH is not altered much by external pH, permeabilities of cell membranes to diffusion of NO_3^- can be affected (Raven & Smith, 1980). Incubation pH influences NO_2^- production during NR assays in vivo of triticale (Lin & Kao, 1980). The incubation pH needed to maximise NO_2^- production during NR assays in vivo is species-dependent in the range pH 6-10 (Jaworski, 1971; Lin & Kao, 1980 and Subbaiah & Manikandan, 1983).

e) Incubation temperature

According to Perez & Kliewer (1978), NO_2^- production by grape leaves during NR assays increased to a maximum as incubation temperature increased, and then declined rapidly at supra-optimal incubation temperatures. Also, the rate of degradation or inactivation of spinach NR increased as temperature increased above 30^oC (Magalhaes et al., 1976). The incubation temperature required to maximise NO_2^-

production is species-dependent in the range 30-50°C (Chopra, 1983 and Sylvester-Bradley, 1984), although some workers use an arbitrary 20 or 25°C incubation temperature (Jaworski, 1971 and Jones & Sheard, 1977).

f) Rate of NO_2^- accumulation

Some workers have found NO_2^- to be released from tissues at a linear rate for at least 3 hours during NR assays *in vivo* (Perez & Kliewer, 1978 and Lawrence & Herrick, 1982). Others have detected a lag-phase of 10-60 minutes (depending on plant species and technique) before NO_2^- release becomes linear (Wallace, 1975; Blahova & Segeta, 1980; Timpo & Neyra, 1983 and Gebauer *et al.*, 1984). The rate of NO_2^- release from barley and maize tissues has been observed to decrease with time and eventually NO_2^- production ceases as substrate or reductant for NR becomes exhausted (Ferrari *et al.*, 1973).

g) Leaf slice size

Diffusion of NO_2^- from plant tissues into the incubation medium has been proposed to occur mainly through cut edges of leaves, since the width of leaf slices affected amounts of NO_2^- produced during NR assays *in vivo*. Smaller slices produce more NO_2^- per unit weight and time than larger ones, although if leaves are too finely chopped, partially macerated or squashed, inhibitors of NR are released from maize (James & Smith, 1976) and wheat leaves (Sylvester-Bradley, 1984), and NO_2^- production consequently declines.

2.4.2 Other factors affecting NO_2^- accumulation

a) Pre-incubation

Endogenous NO_2^-

Since the rate of NiRA is usually very much higher than NRA, NO_2^- accumulation in growing plants is rare (Guerrero et al., 1981). Only traces of endogenous NO_2^- have been detected in maize, wheat and barley leaves prior to NR assays (Jones & Sheard, 1977). NO_3^- reduction has been suggested to be the rate-limiting step in the conversion of NO_3^- to NH_4^+ since NO_2^- does not accumulate in growing plants under normal conditions (Beevers & Hageman, 1969 and Klepper, 1979). Free NO_2^- is very toxic to plants (Birch & Eagle, 1969 and Naik & Nicholas, 1984), acting by combining irreversibly with haeme proteins (Mahler & Cordes, 1966).

Time between sampling and assay

Wheat leaf samples have been stored (on ice) for up to 8 hours without appreciable loss of NRA (Jones et al., 1981) but Sylvester-Bradley (1984) noted marked decreases in wheat and barley NRA within 4 hours when material was stored at room temperature.

Vacuum infiltration

Infiltrating incubation media under vacuum into plant tissues prior to assays in vivo for NRA has been found to enhance NO_2^- production by up to 40% (over controls) in barley and soybean (Aslam et al., 1981) and Rumex leaf samples (Gebauer et al., 1984).

Freeze/thaw treatment

Freezing plant tissues in liquid nitrogen prior to assays for NRA has been proposed to increase NO_2^- production by rupturing cell membranes and enhancing diffusion of NO_3^- and NO_2^- in various species (Ferguson and Sims, 1974 and Rhodes & Stewart, 1974). In contrast, other workers have discovered large losses in NRA of spinach (Mann et al., 1979), sorghum (Kadam et al., 1980) and Rumex (Gebauer et al., 1984) after freeze/thaw treatment, possibly due to disruption of mitochondria causing loss of NADH production needed for NO_3^- reduction (Heber & Santarius, 1964).

b) During incubation

Bacterial and non-enzymic NO_2^- production

Bacteria have been proposed to contribute to NO_2^- accumulation during NR assays of barley roots (Blevins et al., 1976) although, in contrast to this, Sanderson & Cocking (1964) showed NRA of non-sterile tomato roots to be similar to that of sterile ones.

No non-enzymic NO_2^- production (by chemical NO_3^- reduction or by chemical NH_4^+ oxidation) has been observed in citrus (Bar-Akiva & Sternbaum, 1965), Lolium (Bowerman & Goodman, 1971) or in maize tissues (Srivastava, 1974).

Anaerobiosis

Removal of oxygen has been suggested to prevent reduction of NO_2^- produced during NR assays (Jones & Sheard, 1978). Many workers have created anaerobic conditions by flushing with nitrogen gas during incubation (Ben-Zioni & Heimer, 1977 and Klepper et al., 1971) and this technique was found to increase NO_2^- accumulation during NR assays

of cotton (Radin, 1973) and barley (Blevins et al., 1976).

In contrast, Canvin & Atkins (1974) showed, (using ^{15}N labelling) that NO_2^- was not reduced under aerobic or anaerobic conditions. In addition, nitrogen flushing was found to give no increases in NO_2^- production during NR assays of soybean (Jaworski, 1971) and wheat tissues (Hageman, 1982).

Light

When NR assays in vivo of wheat, tomato or spinach leaves are carried out in the light, some (or all) of the NO_2^- produced is lost by reduction, catalysed by NiR (Randall, 1969 and Mann et al., 1979). Accordingly, NR assays of leaves are usually performed in darkness to prevent rapid NO_2^- reduction (Klepper et al., 1971) by stopping production of reductant (Fd_{red}) for NiR (Joy & Hageman, 1966).

Fd_{red} has never been detected in roots (Beevers & Hageman, 1972) and the source of reductant for NiR in roots is not known for certain, but is probably provided indirectly by respiration of photosynthates translocated from shoots (Beevers & Hageman, 1969).

NO_2^- reduction during dark assays

Although Canvin & Atkins (1974) demonstrated that NO_2^- reduction was strictly light-dependent, several workers have observed some NO_2^- reduction during dark NR assays in vivo (Jones & Sheard, 1978 and Mann et al., 1979). Beevers & Hageman (1969) proposed that some reductant (Fd_{red}) for NiR could be generated in situ by NAD(P)H in leaves in darkness.

NH_4^+

Addition of NH_4^+ to incubations has been observed to stimulate

NRA of maize (Hageman, 1983), Paul's scarlet rose (Mohanty & Fletcher, 1976) and Pisum (Sihag et al., 1979). Other workers, however, have shown NH_4^+ to decrease NO_2^- production during assays by inhibiting or repressing NR of barley (Smith & Thomson, 1971), cotton (Radin, 1973) and Pisum (Sahulka, 1977).

NADH

NADH has been reported to be the source of reducing power for higher plant NR by Sanderson & Cocking (1964), Shen (1972) and Kadam et al., (1980). When reductant was limiting NRA, the addition of NADH to assays caused increases in NRA of wheat (Brunetti & Hageman, 1976) and Vigna mungo (Srivastava et al., 1982), although the large size of the NADH molecule hindered its transport across cell membranes (Klepper et al., 1971 and Khan et al., 1984).

Excessive amounts of NADH were noted to interfere with NO_2^- determination by the Griess-Ilosvay method (Bowerman & Goodman, 1971 and Nicholas et al., 1976).

Addition of NADH caused no increases in NRA when other factors, such as substrate or carbohydrate were limiting NO_3^- reduction (Nicholas et al., 1976).

Sugars

NRA is coupled to sugar catabolism and carbohydrate metabolism and addition of sugars to NR assays has been shown to increase NRA of Pisum (Sihag et al., 1979) and beans (Puranik & Srivastava, 1983). Sugars only increased NRA in dark-treated (carbohydrate deficient) soybean (Tingey et al., 1974), barley (Aslam et al., 1976) and wheat plants (Simmons & Moss, 1978). Sugars do not enhance NRA in plants kept in the light since some factor (e.g. substrate) other than

carbohydrate supply limits NRA (Tingey et al., 1974 and Simmons & Moss, 1978).

L-malate

L-malate is broken down in plant cells by L-malate dehydrogenase to produce NADH which then increases NO_2^- production when reductant is limiting during NR assays of maize (Neyra & Hageman, 1976), barley (Deane-Drummond & Johnson, 1980), spinach (Mulder et al., 1959) and soybeans (Nicholas et al., 1976). In contrast, L-malate addition had no effect on NO_2^- production during NR assays of several other species (Srinivasan et al., 1982 and Hipkin, 1984), where substrate (and not reductant) probably limited NRA (Hageman et al., 1980).

c) Post-incubation

Enzyme leakage from tissues

NO_2^- production could continue during the colour development period of NO_2^- analysis if active NR (and reductant) contaminated the medium which is removed for NO_2^- analysis after incubation. However, this phenomenon has not been observed and work has shown that all NO_3^- reduction takes place within intact cells and that no active NR leaks out of plant tissues into the incubation medium (Klepper et al., 1971 and Heuer & Plaut, 1978).

NO_2^- retention within tissues

Much NO_2^- remains within wheat leaf tissues after NR assays in vivo, leading to underestimations of NRA when only incubation medium is analysed for NO_2^- (Srinivasan et al., 1982). This retained NO_2^-

can be readily released by boiling samples after incubation (Srinivasan et al., 1982). By contrast, Hog et al., (1983) showed that incubation medium analysis detected about 95% of the NO_2^- produced during NR assays in vivo of wheat leaves.

2.5 Effects of plant factors and environmental conditions on NRA

A wide range of plant factors (e.g. tissue age, position on plant) and environmental conditions (e.g. light, temperature, nutrient pH, form and concentration of nitrogen supplied) influence the capacity of plants to reduce NO_3^- and hence affect NRA (Beevers & Hageman, 1969).

2.5.1 Plant factors

a) Species and variety

Differences in NRA between plant species have been detected by Lillo & Henriksen (1984) using wheat, oats and barley. Also, many workers have observed marked differences in NRA between varieties of a species, e.g. wheat (Dalling et al., 1975), beans (Felix et al., 1981), sudangrass (Eck & Hageman, 1974) and barley (Bowerman & Goodman, 1971; Eck & Hageman, 1974 and Goodman et al., 1974). Varieties of maize differ in seasonal mean NRA by a factor of up to five (Zieserl et al., 1962), while Schrader et al., (1966) showed that hybrids could be developed with a specified high or low NRA. In contrast, only small differences in NRA were noted between wheat and barley varieties with otherwise very different characteristics by Croy & Hageman (1970) and Dale (1976).

b) Leaf position

Highest NRA has been observed in youngest leaves of cucumber

(Blahova & Segeta, 1980), Pisum (Wallace & Pate, 1965), soybean (Harper & Hageman, 1972 and Hatam, 1978) and barley plants (Chatterjee et al., 1980). Other workers however, have discovered NRA to be highest in the older, first formed, leaves of Lolium (Smith & James, 1982), wheat (Kumar et al., 1981) and barley plants (Naik et al., 1982).

c) Leaf age

Leaf NRA increases gradually to a maximum, and then declines rapidly as leaves of cucumber and bean plants develop and subsequently senesce (Franco et al., 1979 and Blahova & Segeta, 1980). However, Smith & James (1982) found little decline in NRA as leaves of Lolium senesce.

d) Part of leaf assayed

NRA has been observed to be greatest in distal parts of wheat (Hallam & Blackwood, 1979) and barley leaves (Jordan & Huffaker, 1972). However, Mack et al., (1984) noted NRA to be highest in the proximal tissues of maize leaves.

2.5.2 Light conditions

a) Light intensity

Many workers have suggested that photosynthesis is essential for induction of NR in higher plants (Hageman & Flesher, 1960; Kannangara & Woolhouse, 1967 and Travis et al., 1970). Light has been proposed to stimulate NO_3^- uptake, to promote transfer of NO_3^- from storage to metabolic pools, to be required for NR synthesis (or to activate pre-existing NR) and to provide reductant (NADH) for NO_3^-

assimilation (Aslam et al., 1976 and Naik et al., 1982).

The light intensity under which plants are grown affects NO_3^- uptake rates and NRA of maize and radish plants (Beevers et al., 1965) and of soybeans Nicholas et al., 1976). NRA is greater at higher light intensities in barley (Felippe et al., 1975), Glycine max (Nicholas et al., 1976) and in Pisum, wheat and maize leaves (Jones & Sheard, 1977). It has been proposed that greater light intensities increase NO_3^- uptake, induce NR and hence produce higher NRA (Beevers et al., 1965; Beevers & Hageman, 1972 and Rao & Rains, 1976).

A linear decline in NRA of maize leaves as light intensity decreased has been observed by Hageman & Flesher (1960). Other workers however, have found that about 15% of maximum light intensity was capable of supporting 50% of the NRA obtained using maximum light intensity for Lolium (Bowerman & Goodman, 1971) and for barley leaves (Jones & Sheard, 1977). Also, a log-linear relationship has been noted between light intensity and NRA of Lolium (Smith & James, 1982).

b) Diurnal variation

A marked diurnal variation in leaf NRA has been detected in cotton (Bilal & Rains, 1973), Lolium (Bowerman & Goodman, 1971), soybeans (Harper & Hageman, 1972) and maize (Robin, 1979). In general, NRA is low at the start of the photoperiod and increases to a maximum just after mid-photoperiod before decreasing thereafter (Wallace & Pate, 1965). Little diurnal variation in NRA of Lolium and Agrostis was found by Smith & James (1982) and Harris & Whittington (1983) respectively.

c) Darkness

Rapid declines in NRA were seen when radish, barley, maize and Perilla plants were placed in darkness (Beevers et al., 1965; Kannangara & Woolhouse, 1967 and Travis et al., 1970). NRA of maize and barley leaves decreased by about 50% after 24 hours in darkness (Hageman & Flesher, 1960 and Travis et al., 1969) while NRA of maize declined to virtually zero after 3-4 days of darkness (Hageman & Flesher, 1960). Beevers & Hageman (1969) and Aslam et al., (1979) proposed that lack of carbohydrates and reductant (NADH) in plants kept in darkness caused NRA to decrease, while Travis et al. (1969) suggested that inhibitors of NR accumulate in the dark.

d) Light after a long dark period

When etiolated seedlings are transferred to the light, and supplied with NO_3^-, NR is induced progressively (Srivastava, 1980). NR starts to increase within 3-6 hours of returning etiolated rice and barley plants to light (Travis et al., 1969 and Sawhney & Naik, 1972) and maximal induction of NR is observed after 24-48 hours (Travis et al., 1969).

2.5.3 Nitrogen nutrition

a) NRA of plants grown without NO_3^-

NR is substrate-inducible (adaptive) and its activity has been shown to be positively related to NO_3^- availability or concentration (Beevers & Hageman, 1969; Wray & Filner, 1970 and Aslam et al., 1973). NO_3^- has been found to be essential for induction and maintenance of NRA (Schrader et al., 1968 and Zeilke & Filner, 1971). Hence, no NRA_e

has been detected in cauliflower (Afridi & Hewitt, 1964), soybean (Aslam, 1982), Phaseolus (Streit & Feller, 1982) or barley plants (Somers, 1983) grown in NO_3^--free media. Shen (1969) and Lee & Stewart (1978) however, have detected a low "background" NRA_e in many plant species when grown without NO_3^-.

b) Induction by NO_3^-

Hageman & Flesher (1960), Sanderson & Cocking (1964) and Aslam et al., (1976) have shown that NR of maize, tomato and barley plants is adaptive in that activity is induced when NO_3^- is supplied. Rapid NR induction can be seen within a few hours after supplying NO_3^- to Pisum (Wallace & Pate, 1965), barley (Dale et al., 1974) and numerous other species (Afridi & Hewitt, 1962 and Lee & Stewart, 1978). Maximal substrate induction of NR has been observed 24-72 hours after supplying NO_3^- to wheat (Brunetti & Hageman, 1976), barley (Somers et al., 1983) and spinach plants (Kaplan & Lips, 1984). It has been suggested that NO_3^- brings about induction of de novo NR protein synthesis rather than activation of pre-existing enzyme (Hageman & Hucklesby, 1971 and Zeilke & Filner, 1971).

c) Withdrawal of NO_3^- nutrition

The presence of NO_3^- in the rooting medium and a continuous flux to the leaves has been reported to be essential for induction and maintenance of high NRA in maize (Shaner & Boyer, 1976) and tobacco (Zeilke & Filner, 1971). When NO_3^- supplies are withdrawn, NRA has been noted to decline rapidly within 1-3 days in Lolium (Bowerman & Goodman, 1971), spinach (Kaplan & Lips, 1984) and Rumex (Melzer et al., 1984). By contrast, Hewitt (1982) noted that barley NRA remained at

initial levels for 2 days after removal of NO_3^- nutrition before declining.

d) NO_3^- concentration

The NO_3^- concentration in the rooting medium needed to saturate induction of NR (i.e. produce maximal activity) has been found to be dependent on plant species and growing conditions (Beevers et al., 1965 and Srivastava, 1980). NO_3^- concentrations in the range of 7-1400 mg N l^{-1} have been needed to produce maximal induction of NR in Lolium (7 mg N l^{-1}) (Bowerman & Goodman, 1971), wheat (140 mg N l^{-1}) (Brunetti & Hageman, 1976), maize (1400 mg N l^{-1}) (Wallace, 1973) and numerous other plant species (Srivastava, 1980).

e) Form of nitrogen

Ammonia is utilised preferentially from complete nutrient solutions containing NH_4NO_3 by many plant species (Shen, 1969; Pate, 1973; Frith & Nicholas, 1975 and Rao & Rains, 1976). Also, Guerrero et al., (1981) showed that NO_3^- uptake from NH_4NO_3 solutions started only after NH_4^+ was exhausted, or its concentration greatly depleted, and that NH_4^+ inhibited NO_3^- uptake.

More NH_4^+, than NO_3^-, was taken up by plants when these forms of N were supplied individually in complete nutrient solutions (Breteler & Smit, 1974), and this was reflected in higher dry weights (Smith & Rice, 1983) and greater total N concentrations of NH_4^+ - than of NO_3^- - fed plants (Harada et al., 1968 and Blair et al., 1970). Reduction of NO_3^- to useable NH_4^+ requires the donation of 8 electrons, and Beevers & Hageman (1969) proposed that energy saved by utilising NH_4^+ (rather than NO_3^-) could be used to produce extra growth.

NRA has often been found to be highest in plants supplied with NO_3^-, intermediate in plants fed NH_4NO_3 (Pate, 1973) and very low (Fair et al., 1974 and Ferguson & Knypl, 1974) or zero in those supplied with NH_4^+ (Afridi & Hewitt, 1964 and Weissman, 1972). Several workers have proposed that NH_4^+ is an end-product inhibitor of NR (Smith & Thomson, 1971; Stewart, 1972 and Orebamjo & Stewart, 1975) and that NH_4^+ inhibits NO_3^- uptake (Srivastava, 1980).

Other workers though, have not detected any repression of NR by NH_4^+ (Bowerman & Goodman, 1971) while several have even found NH_4^+ to promote NR (Schrader & Hageman, 1967; Bayley et al., 1972 and Mohanty & Fletcher, 1976).

2.5.4 Nutrient solution pH

Although intracellular pH is not altered much by external pH, changes in external pH can alter rates of NO_3^- uptake via ion antagonism and by affecting permeabilities of cell membranes (Raven & Smith, 1980). More NO_3^- has been found to be taken up by plants grown at lower pH (Rao & Rains, 1976), and higher shoot NRA was detected in tomato plants grown at pH 4.5 than at pH 6.5 (Iken & Marcus-Wyner, 1984).

2.5.5 Temperature

NR has been shown to be under the control of a turnover system, with levels of activity being governed by relative rates of enzyme synthesis and/or activation and of degradation or inactivation (Beevers & Hageman, 1969 and Travis et al., 1969). When plants were grown at different temperatures, these rates of synthesis/activation and degradation/inactivation were found to be altered differentially.

Thus, leaf NRA was observed to be greater at lower, than at higher, temperatures in species of pasture grasses (Harmer & Lee, 1981), Agrostis (Harris & Whittington, 1983) and Sinapis (Moroz et al., 1984). Similarly, transfer of cucumber plants from a temperature of $2^{o}C$ to $20^{o}C$ caused a decline in NRA (Blahova, 1982) and tomato root NRA decreased as root temperature increased from $12^{o}C$ to $30^{o}C$ (Gosselin et al., 1984).

2.6 Practical applications of NRA measurements

NRA levels have been proposed to give estimates of cereal plant N status and to be positively correlated with growth, dry matter production, grain yields and protein contents (Croy & Hageman, 1970; Eck et al., 1975; Srivastava, 1980; Hageman, 1982 and Nair and Abrol, 1982). NRA_e and NRA_i levels have also been used as indicators of plant N status for cauliflower (Jungk & Wehrmann, 1978), cotton (Oosterhuis & Bate, 1983) and citrus crops (Bar-Akiva & Sternbaum, 1965).

NRA levels have been used to determine the most suitable time to apply N fertiliser to maximise crop utilisation and increase yields. Patriquin (1978) proposed that N fertiliser was utilised most effectively by maize plants if it was applied when NRA was high; while Kapoor & Li (1982) applied N fertiliser when NRA levels of potato plants started to decline from their maximum values.

Nitrate assimilation capacity, NAC ($= {}^{NRA}i/NRA_e$) has also been used successfully as an indicator of N status of citrus trees (Shaked et al., 1974), Laminaria (Davison & Stewart, 1983) and cereal crops (Verstraeten, 1983), although Sylvester-Bradley (1982) thought NRA was too labile to be of use as an indicator of field crop N status.

3. Assay in vivo of NR in barley

3.1 Introduction

NR assays *in vivo* are carried out using excised, fresh plant material. Assays for endogenous NRA (NRA_e) rely on endogenous substrate (NO_3^-) and reductant (NADH) for utilisation by NR, which catalyses reduction of NO_3^- to NO_2^- during a period of dark incubation. The rate of NO_2^- accumulation is then taken as a measure of NRA_e (Jaworski, 1971). If NO_3^- is added to the incubation medium, a higher NRA may be induced, NRA_i (Bar-Akiva *et al.*, 1970 and Chopra, 1983).

NR assays *in vivo* have many advantages over assays *in vitro*. NR assays *in vivo* involve only the use of a guillotine to chop plant material and one incubation medium (Sylvester-Bradley, 1984), allowing as many as 70 samples to be assayed within 3 hours. By contrast, assays *in vitro* require more equipment (homogeniser, centrifuge), expensive reagents and prohibitively long preparation times if many samples are involved.

NR activities measured in assays *in vivo* may also have the advantage of reflecting rates of NO_3^- reduction in growing plants better than activities measured in assays *in vitro* (Verstraeten, 1983 and Brunetti & Hageman, 1976), since substrate or reductant supply probably limits NRA *in vivo*. In assays *in vitro*, by contrast, substrate and reductant are usually present in excess and only the concentration of NR limits NRA. Hence, much higher NRA levels are usually detected in assays *in vitro*, compared to assays *in vivo*, and these high levels may overestimate true NO_3^- reduction rates in growing plants.

Due to the advantages outlined above, NR assays *in vivo* were

therefore used throughout this work.

3.2 Standard plant culture

Barley seeds (Hordeum vulgare L. cultivar Igri) were germinated on netting stretched 1cm above 10^{-4}M $CaSO_4$ solution and kept moist until roots grew into the solution. When seedlings were about 3cm tall, they were removed and supported individually with paper in holes in boards over trays of complete nutrient solution containing (mg l^{-1}):

K	140	Mg	15	Mn	1	Zn	0.1
Ca	60	NO_3^--N	10	B	0.3	Mo	0.02
P	25	Fe	2.5	Cu	0.1		

Solutions had a conductivity of 1000 $\mu S\,cm^{-1}$, a pH of 6.3 and were contained in 40 x 25 x 7.5cm trays, each holding 6 litres. Each tray supported 40 plants and solutions were replaced every 3 days.

Plants were grown in a glasshouse with maximum/minimum temperatures of 25/15°C. They were shaded from sunlight and given 16hd^{-1} supplemental lighting of average intensity 5klx until they reached the 3rd leaf stage, Zadoks growth stage 13 (Tottman & Makepeace, 1979). Leaves and entire root systems of 25 plants from each tray were then harvested for use as standard material in the experiments described in section 3.4.

3.3 NR assay in vivo procedure

Leaves and roots were chopped into 10mm lengths, bulked separately and sub-sampled to provide uniform material for NRA_i determination. 500mg samples of leaves or roots were incubated in the dark in 5ml of a medium containing phosphate buffer, organic

solvent, surfactant and KNO_3. KNO_3 was omitted from the medium if NRA_e was to be determined.

Samples were incubated for 1 hour in a heated, shaking water bath and then 1ml of medium was withdrawn for NO_2^- analysis by the Griess Ilosvay method (Appendices 1-6). Before measuring absorbances of the dye formed in the Griess Ilosvay reaction, samples were centrifuged to remove turbidity caused by cell fragments produced during tissue chopping. Absorbances of sample blanks were then subtracted to account for green chlorophyll pigments extracted into the medium by the surfactant and organic solvent present.

Finally, NRA was expressed as the number of micromoles of NO_2^- produced per gram fresh weight of plant tissue per hour, i.e. μmol NO_2^- g^{-1} fresh weight h^{-1}.

All replicated results obtained during this work were analysed using standard statistical methods (Appendix 12).

3.4 Optimisation of the assay in vivo for NR in barley (variety Igri).

3.4.1 Introduction

Optimum conditions for NR assays *in vivo* differ between species, and sometimes between varieties and plant organs (Jones & Sheard, 1977; Perez & Kliewer, 1978; Lin & Kao, 1980 and Sylvester-Bradley, 1984). By optimising assay conditions, NO_2^- production can be maximised, leading to enhanced assay sensitivity and reproducibility. Optimum conditions for assays *in vivo* of NR in leaves and roots of barley (variety Igri) were therefore determined by adjusting the basic procedure (section 3.3) to maximise NO_2^- production. Factors investigated included:

a) Solvents (section 3.4.2);

b) Surfactant (section 3.4.3);

c) Exogenous NO_3^- (section 3.4.4);

d) Phosphate buffer (section 3.4.5);

e) Incubation pH (section 3.4.6);

f) Incubation temperature (section 3.4.7);

g) Rate of NO_2^- accumulation (section 3.4.8);

h) Leaf slice size (section 3.4.9);

i) Miscellaneous factors affecting NO_2^- accumulation before, during and after incubation (section 3.4.10).

The optimisation of the NR assay in vivo for barley (variety Igri) described here (section 3.4) was published as a research paper (Sym, 1984, Appendix 15).

3.4.2 Solvents

Organic solvents were found to increase NO_2^- production when added to incubation media during NR assays in vivo (Jaworski, 1971). Propan-1-ol is commonly used, although Jones & Sheard (1977) showed that the choice, and concentration, of solvent to maximise NO_2^- production was species dependent. Accordingly, the concentration of propan-1-ol required to maximise NO_2^- production during NR assays in vivo of barley (variety Igri) was determined, while in a second experiment the solvents acetone, methanol, propan-2-ol and ethanol were compared with propan-1-ol.

Standard leaves and roots (section 3.2) were assayed for NRA_i in media containing 0 to 5% (v/v) propan-1-ol, 0.05 M phosphate buffer at pH 7.5 and 0.02 M KNO_3.

Leaf and root NRA_i were enhanced most by 3% (v/v) propan-1-ol

(Figure 1) and this concentration was therefore deemed optimum for subsequent use. Other workers have observed optimum propan-1-ol concentrations in the range 0.5 to 5% (v/v) depending on plant species (Jaworski, 1971 and Jones & Sheard, 1977).

In the second experiment, acetone, methanol, propan-2-ol, or propan-1-ol (all at a concentration of 3% (v/v)) were added to incubation medium and leaf and root NRA_i were determined.

Acetone and propan-1-ol enhanced leaf NRA_i to a greater extent than did methanol, ethanol or propan-2-ol, although the effect was not marked in roots (Figure 2). Propan-1-ol was chosen for subsequent use in preference to acetone, since acetone extracted large amounts of green pigments (chlorophylls) into the medium during leaf assays and this interfered with colorimetric NO_2^- detection. Propan-1-ol caused some pigments to diffuse into the medium but interference with NO_2^- detection was avoided by subtracting absorbances of sample blanks (Appendix 1). Streeter & Bosler (1972) observed that propan-1-ol extracted chlorophyll from tissues at an increasing rate as time passed.

Solvents may increase NO_2^- production during NR assays *in vivo* by facilitating NO_3^- diffusion through cell membranes into metabolically available cytoplasmic pools where it can induce NRA.

3.4.3 Surfactant and solvent

Addition of surfactants (wetting agents) to NR assay *in vivo* media has been shown to increase NO_2^- production (Lawrence & Herrick, 1982). In a further attempt to increase NO_2^- production, both solvent and surfactant can be added to incubation media (Nicholas *et al.*, 1976 and Jones & Sheard, 1977). The surfactant concentration needed to maximise NO_2^- production during NR assays *in vivo* was therefore

FIGURE 1

Influence of propan-1-ol concentration in the incubation medium on shoot and root NRA_i

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level

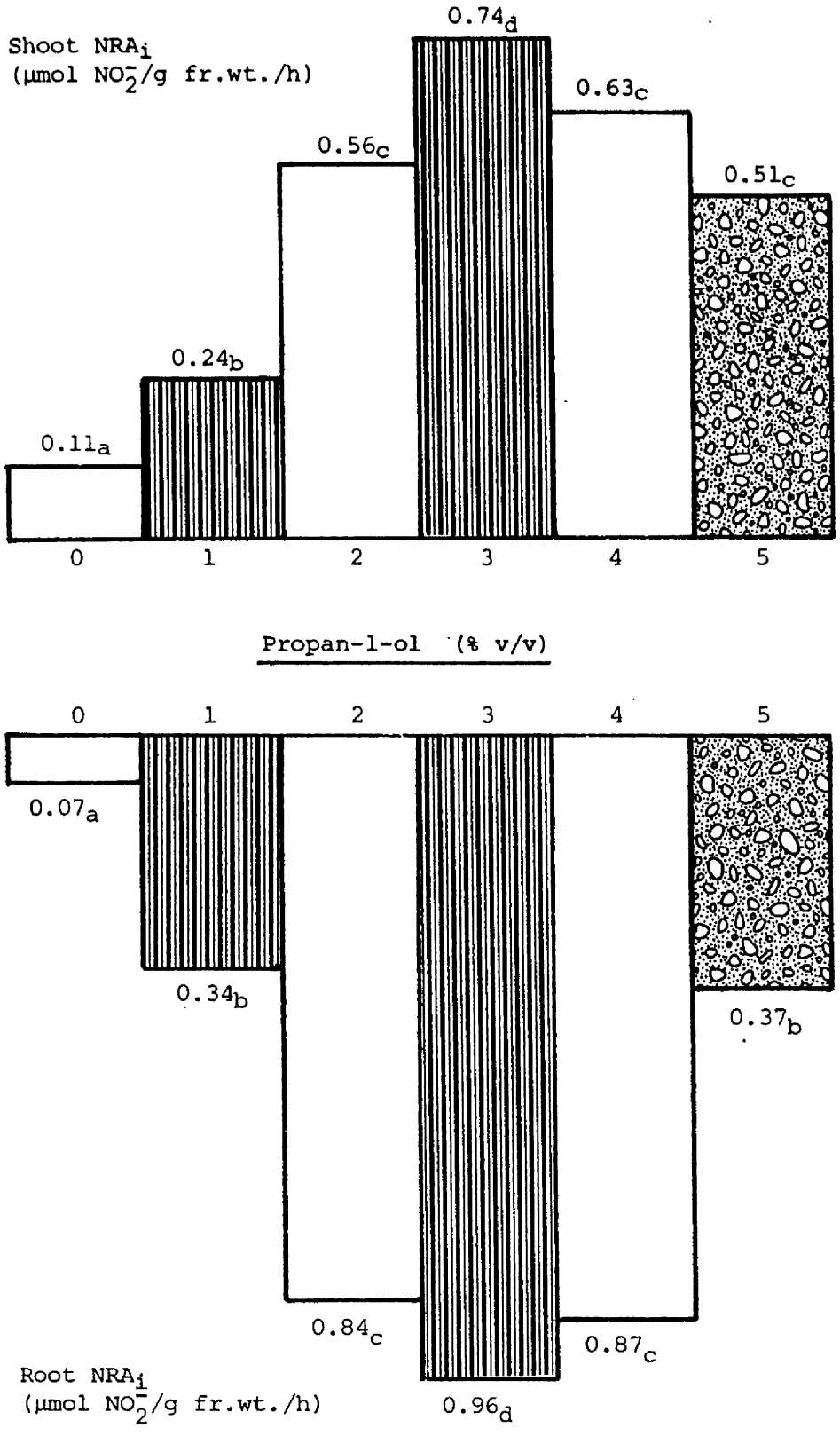
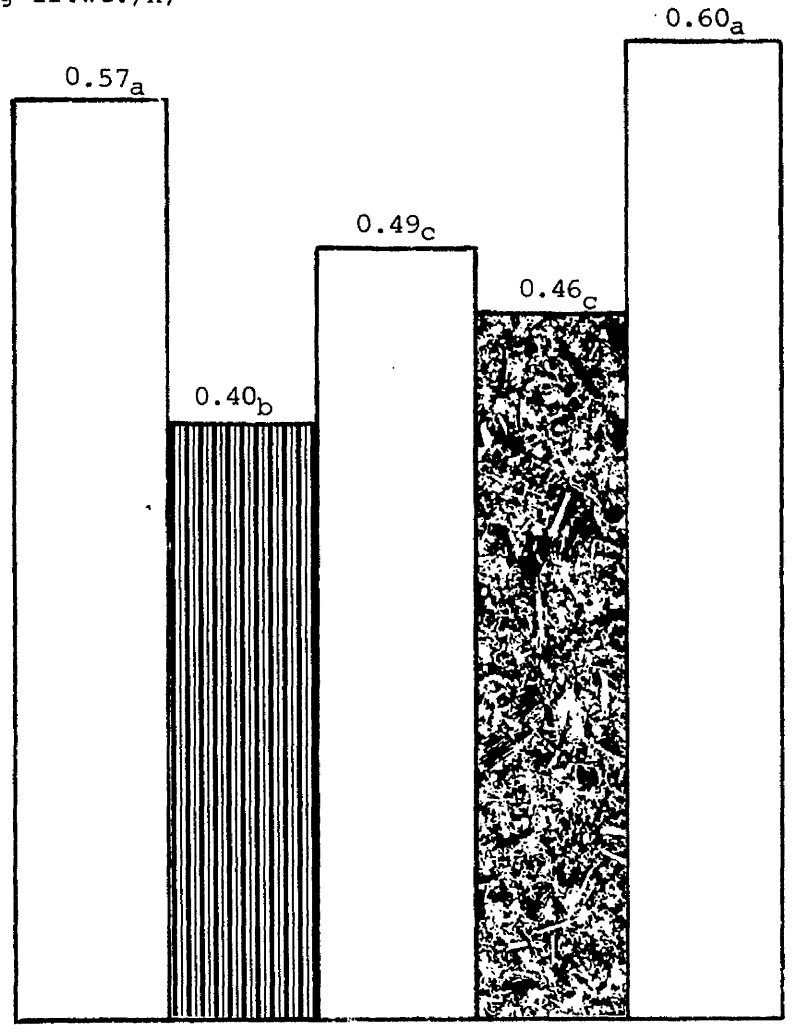

Shoot NRA$_i$
(μmol NO_2^-/g fr.wt./h)
0.11$_a$
0.24$_b$
0.56$_c$
0.74$_d$
0.63$_c$
0.51$_c$
0 1 2 3 4 5
Propan-1-ol (% v/v)
0 1 2 3 4 5
0.07$_a$
0.34$_b$
0.84$_c$
0.96$_d$
0.87$_c$
0.37$_b$
Root NRA$_i$
(μmol NO_2^-/g fr.wt./h)

FIGURE 2

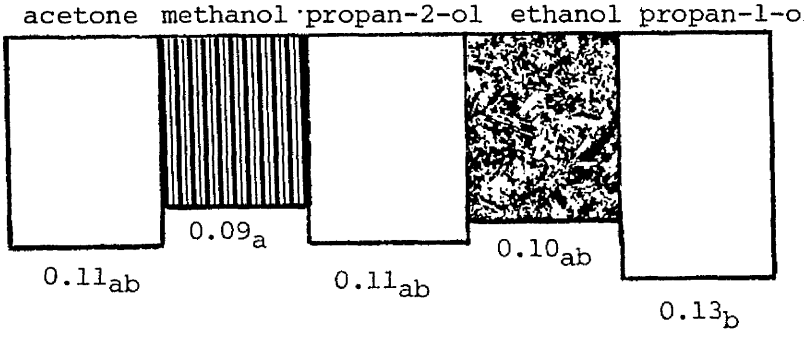
Influence of various organic solvents in the incubation medium on shoot and root NRA_i

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level

Shoot NRA_i
(μmol NO_2^-/g fr.wt./h)



Organic solvent (3% v/v)



Root NRA_i
(μmol NO_2^-/g fr.wt./h)

determined for barley (variety Igri), while in a second experiment, addition of both surfactant and solvent was investigated.

Concentrations of the surfactant Triton X-100, ranging from 0.01 to 0.1 % (v/v), were added to incubation medium containing 0.05M phosphate buffer at pH 7.5 and 0.02M KNO_3. Standard leaves and roots (section 3.2) were then assayed for NRA_i in these media.

Triton X-100 increased shoot and root NRA_i most at a concentration of 0.01% (v/v) (Figure 3), and this concentration was therefore chosen as optimal for subsequent use. However, addition of surfactant caused smaller increases in NRA_i than did propan-1-ol (section 3.4.2). Optimum surfactant concentrations doubled NRA_i, while optimum propan-1-ol concentrations caused 7 fold increases in shoot NRA_i and 13 fold increases in root NRA_i compared with medium containing no surfactant or solvent.

In the second experiment, concentrations of Triton X-100, in the range 0-0.05% (v/v), were added to medium containing 3% (v/v) propan-1-ol, 0.05M phosphate buffer at pH 7.5 and 0.02 M KNO_3. Leaves and roots were then assayed for NRA_i in these media.

Addition of 0.01% (v/v) Triton X-100 to medium containing 3% (v/v) propan-1-ol caused further increases in shoot and root NRA_i compared with the 3% (v/v) propan-1-ol only treatment (Figure 4). Therefore, 0.01% (v/v) Triton X-100 + 3% (v/v) propan-1-ol was chosen as optimum. This combined effect of surfactant and solvent increased NO_2^- production by about 15% over the 3% (v/v) propan-1-ol only treatment and provided a useful increase in assay sensitivity.

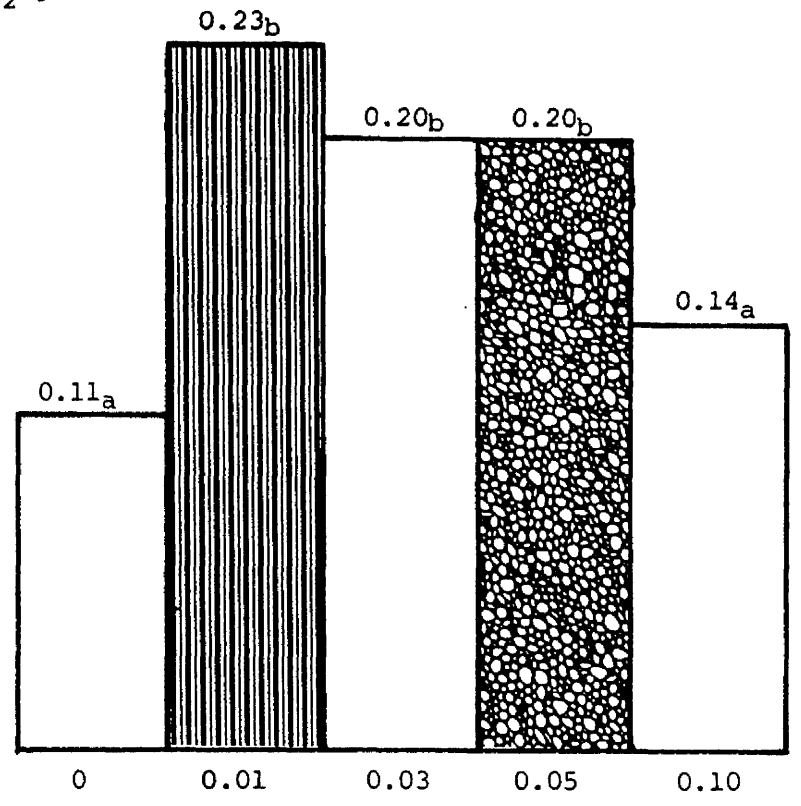
Surfactants probably increase NO_2^- production during NR assays in vivo in a similar manner to solvents (see section 3.4.2).

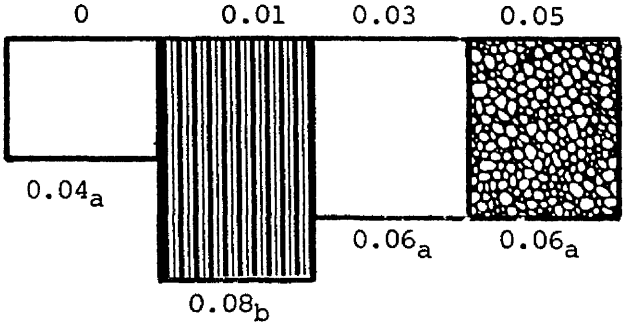
FIGURE 3

Effect of surfactant concentration in the incubation medium on shoot and root NRA_i

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level

Shoot NRA_i
(μmol NO_2^-/g fr.wt./h)





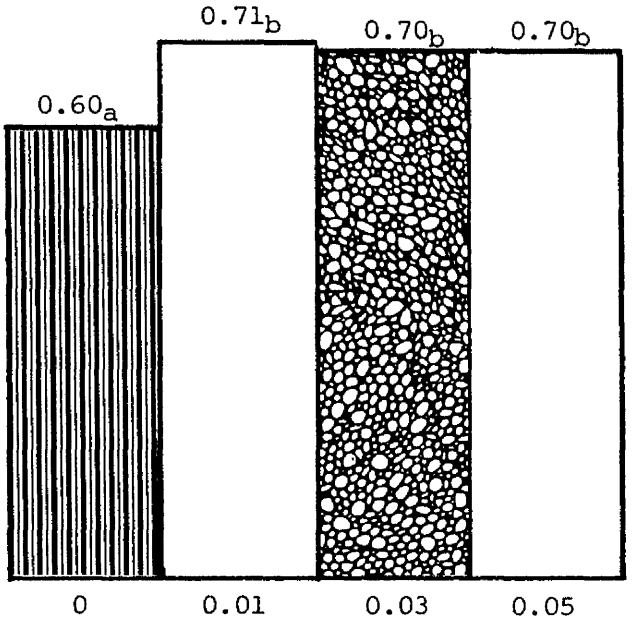
Root NRA_i
(μmol NO_2^-/g fr.wt./h)

FIGURE 4

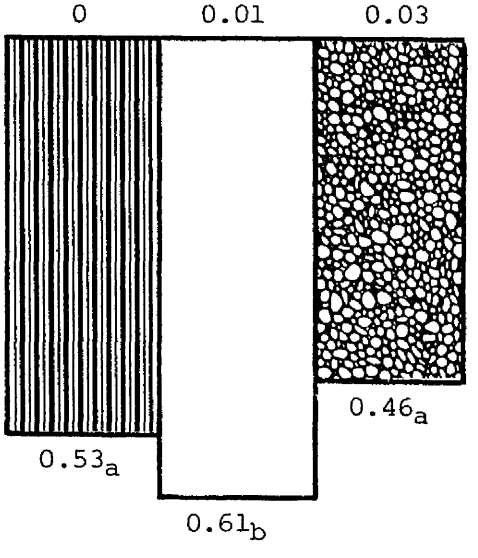
Influence of both propan-1-ol and Triton X-100 in the incubation medium on shoot and root NRA_i

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level

Shoot NRA_i
(μmol NO_2^-/g fr.wt./h)



3% Propan-1-ol + Triton X-100 (% v/v)



Root NRA_i
(μmol NO_2^-/g fr.wt./h)

3.4.4 Nitrate

When exogenous NO_3^- is added to NR assays *in vivo*, NRA is induced (Sylvester-Bradley, 1984). However, the exogenous NO_3^- concentration needed to saturate (produce maximal) induction of NRA was seen to vary between species (Perez and Kliewer, 1978 and Lin & Kao, 1980) and so an experiment was performed to determine the optimum level for barley (variety Igri).

Various concentrations of KNO_3 (final concentrations of 0.01-0.08M) were added to incubation medium containing 3% (v/v) propan-1-ol, 0.01% (v/v) Triton X-100 and 0.05M phosphate buffer at pH 7.5. Leaves and roots from standard plants (section 3.2) were then assayed for NRA_e (in the treatment to which no KNO_3 was added) and NRA_i (in treatments to which KNO_3 was added).

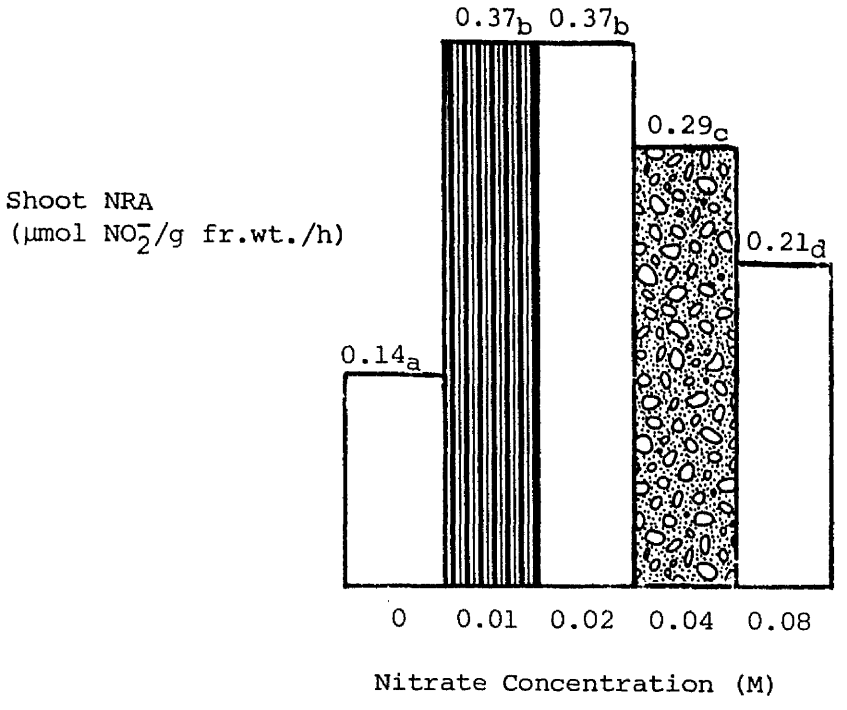
Exogenous KNO_3 concentrations of 0.02M caused large increases in shoot and root NRA over the low NRA_e value (Figure 5). Greater exogenous KNO_3 concentrations caused marked decreases in shoot NRA_i. Hence, 0.02M KNO_3 was chosen as optimal for maximising induction of NRA during a 1 hour assay, which compares to a value of 0.025M found to be optimal for barley by Chatterjee *et al.* (1981).

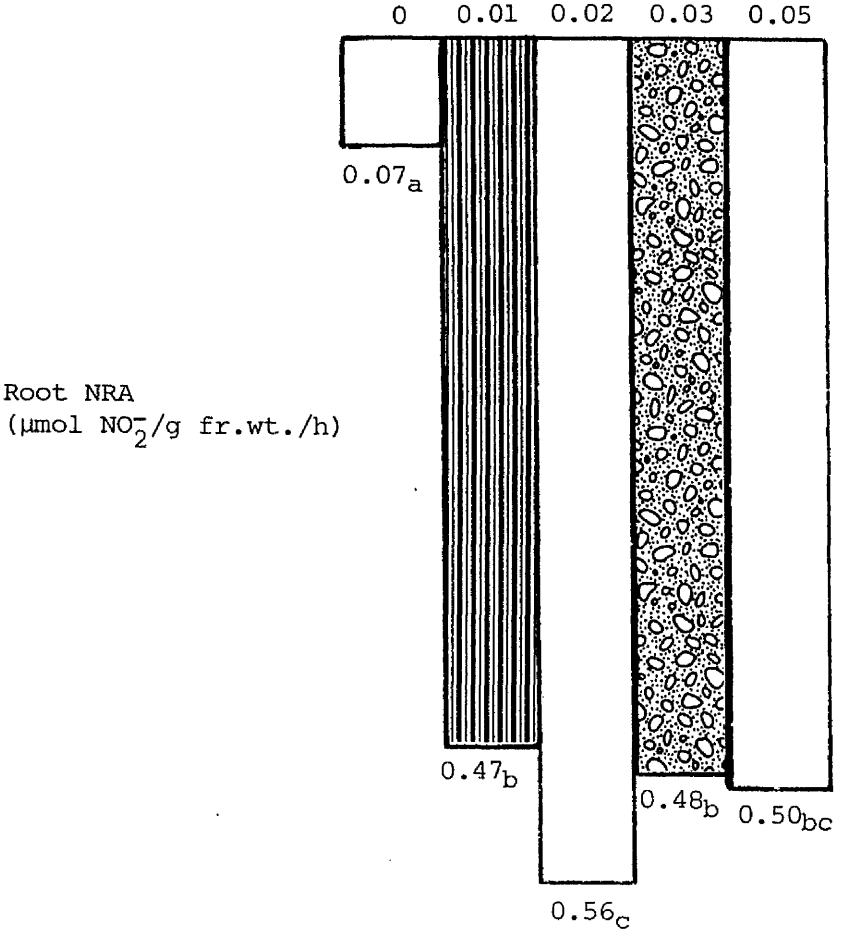
Optimal KNO_3 concentrations have been shown to be species dependent, in the range 0.01-0.3M KNO_3 (Jaworski, 1971; Perez & Kliewer, 1978 and Lin & Kao, 1980). Concentrations of exogenous NO_3^- greater than 0.5M were found to inhibit or repress NR and hence reduce NO_2^- production during NR assays *in vivo* (Jones & Sheard, 1977 and Sylvester-Bradley, 1984).

FIGURE 5

Effect of NO_3^- concentration in the incubation medium on shoot and root NRA_i

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level


Shoot NRA
(μmol NO_2^-/g fr.wt./h)
0.37_b 0.37_b
0.29_c
0.21_d
0.14_a
0 0.01 0.02 0.04 0.08
Nitrate Concentration (M)


Root NRA
(μmol NO_2^-/g fr.wt./h)
0 0.01 0.02 0.03 0.05
0.07_a
0.47_b
0.56_c
0.48_b
0.50_{bc}

3.4.5 Phosphate buffer

Enzymes work most effectively only within a narrow pH range. It is therefore important to use a buffer of sufficient capacity to prevent organic acids, released from plant material, lowering the pH of the incubation medium outside the optimal range during NR assays *in vivo* (Jaworski, 1971). If an excessively high buffer concentration is used, osmotic/ionic strength effects may repress NR (Sylvester-Bradley, 1984). Accordingly, the effect of phosphate buffer concentration in NR assays *in vivo* of barley (variety Igri) was studied.

Phosphate buffer, in the concentration range 0.03-0.15M, was added to incubation medium containing 3% (v/v) propan-1-ol, 0.01% (v/v) Triton X-100 and 0.02M KNO_3. The initial pH of the medium was 7.5 in each case. Shoots and roots of standard plants (section 3.2) were then assayed for NRA_i in incubation media differing in buffer concentration.

TABLE 1: Influence of phosphate buffer concentration in the incubation medium on shoot and root NRA_i

Phosphate buffer concentration (M)	NRA_i (μmol NO_2^- g^{-1} fresh weight h^{-1}) Shoot	Root
0.03	-	0.40_{cd}
0.05	0.37_a	-
0.07	-	0.43_c
0.08	0.37_a	-
0.10	0.37_a	0.43_c
0.15	0.37_a	0.35_d

- : not tested

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

The buffer concentrations tested had little effect on shoot/root NRA_i (Table 1) and 0.1M was chosen for subsequent use to provide a large pH buffering capacity. Other workers also demonstrated that buffer concentration had no effect on NRA if extremely high or low strengths were avoided (Jaworski, 1971; Jones & Sheard, 1977 and Lin & Kao, 1980).

3.4.6 Incubation pH

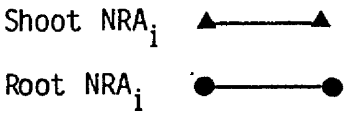
Most workers have observed that incubation pH greatly affects amounts of NO_2^- released during NR assays *in vivo* (Jaworski, 1971 and Subbaiah & Manikandan, 1983). Also, the incubation pH which gave maximum NO_2^- production was seen to vary between species (Lin & Kao, 1980 and Subbaiah & Manikandan, 1983). The optimal incubation pH was therefore determined for barley (variety Igri).

Standard leaves and roots (section 3.2) were assayed for NRA_i in media with pH values in the range 4.5-8.5, containing 3% (v/v) propan-1-ol, 0.01% (v/v) Triton X-100, 0.02M KNO_3 and 0.1M phosphate buffer. Media differing in pH were prepared by adjusting the $KH_2PO_4 : K_2HPO_4$ ratio in the buffer.

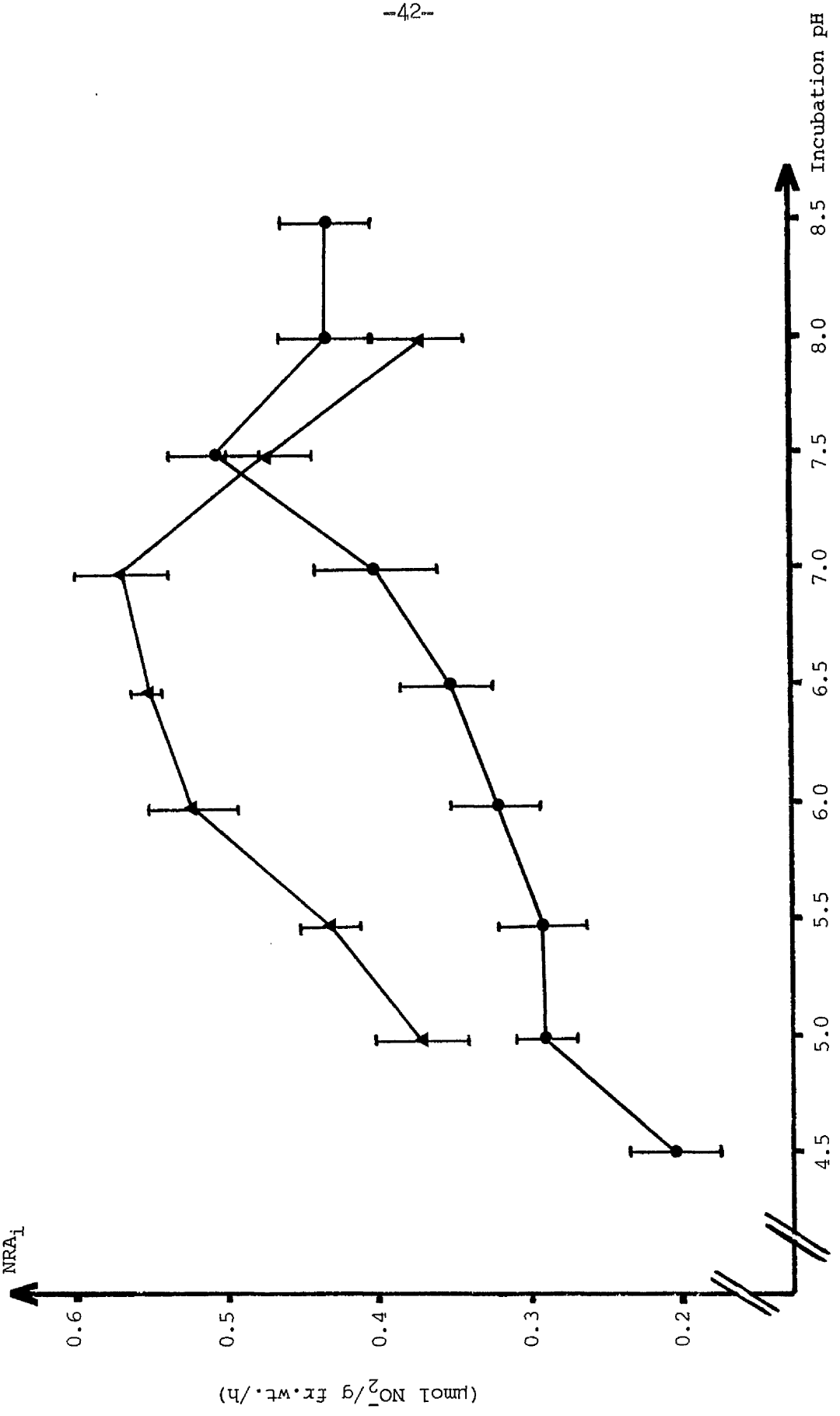
An incubation pH of 6.0-7.0 gave highest shoot NRA_i, while a pH of 7.5 gave highest root NRA_i (Figure 6). Media with pH 7.0 and 7.5 were therefore chosen as optimal for subsequent shoot and root assays respectively. Other workers have demonstrated optimal pH to be in the range pH 6-10 depending on species (Lin & Kao, 1980 and Subbaiah & Manikandan, 1983).

FIGURE 6

Effect of incubation pH on shoot and root NRA_i



Each point represents the mean of 5 replicates
Error bars mark standard deviations of means


NRA$_i$
0.6
0.5
0.4
0.3
0.2
(μmol NO_2^-/g fr.wt./h)
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Incubation pH

3.4.7 Incubation temperature

The incubation temperature at which NR assays are carried out affects the amount of NO_2^- produced (Perez & Kliewer, 1978), and the optimum incubation temperature for maximising NO_2^- production during NR assays *in vivo* was found to be plant species dependent (Chopra, 1983). Many workers have, however, used an arbitrary 20 or 25°C incubation temperature, with a resultant decrease in the potential amount of NO_2^- which could be produced and a concomitant reduction in assay sensitivity (Jaworski, 1971 and Jones & Sheard, 1977). Accordingly, the incubation temperature necessary for maximising NO_2^- production during NR assays *in vivo* was determined for barley (variety Igri).

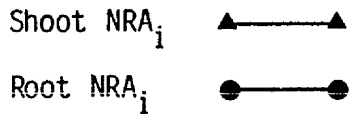
Shoot and root NRA_i were determined using samples of standard plant material (section 3.2) which were incubated at a constant temperature (in the range 17-36°C). The incubation medium contained 3% (v/v) propan-1-ol, 0.01% (v/v) Triton X-100, 0.02M KNO_3 and 0.1M phosphate buffer, at a pH of 7.0 and 7.5 for shoot and root assays respectively.

Shoot and root NRA_i exhibited similar temperature profiles, (Figure 7) with NRA_i increasing to a maximum at incubation temperatures of 31-34°C and then decreasing as NR was degraded or inactivated at higher temperatures. An incubation temperature of 32°C was therefore chosen as optimal for subsequent assays of shoots and roots for NRA_i.

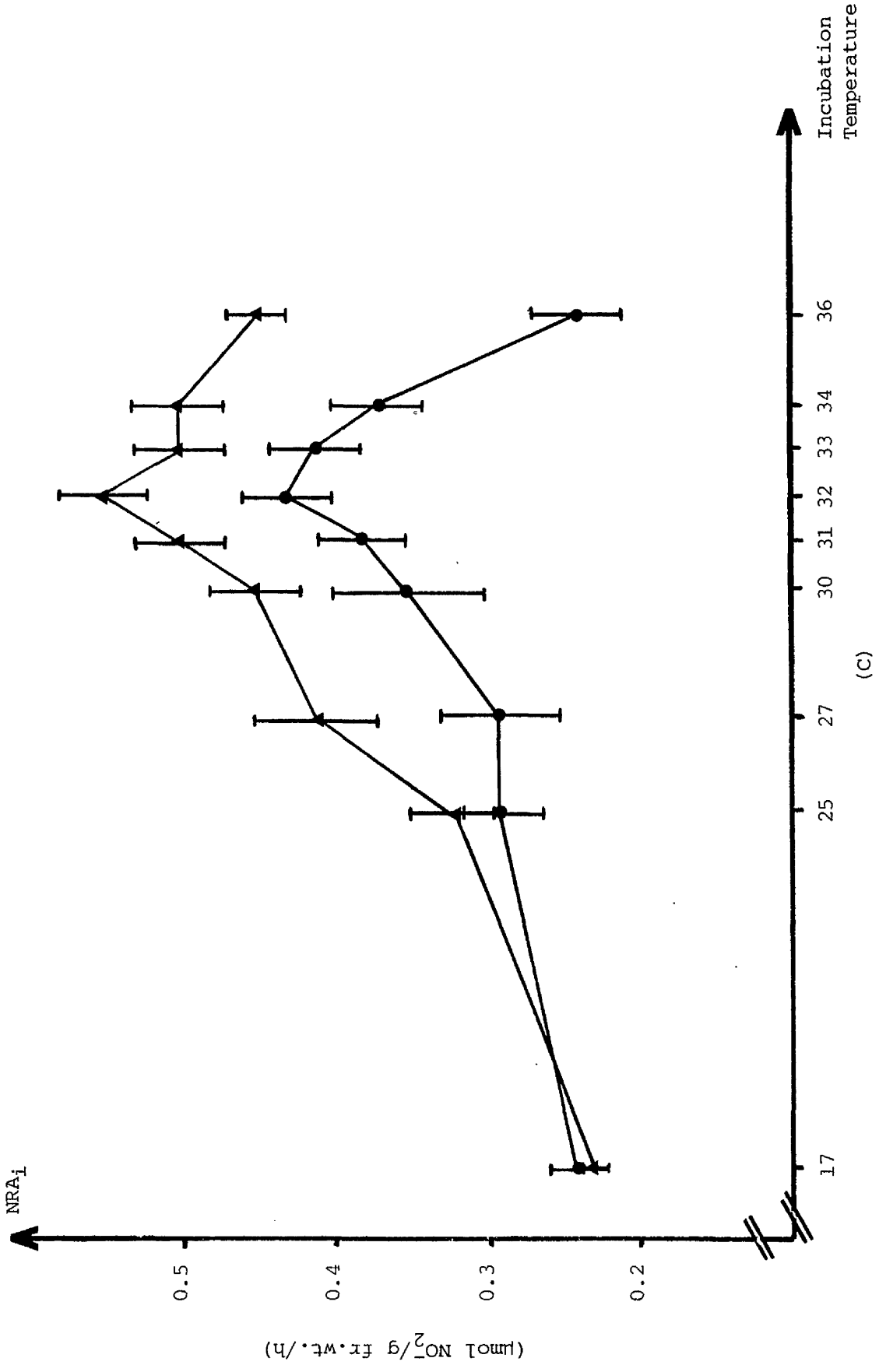
Other workers have discovered similar temperature profiles (Perez & Kliewer, 1978), with NRA being highest in the temperature range 30-50°C depending on species (Chopra, 1983 and Sylvester-Bradley, 1984).

FIGURE 7

Effect of incubation temperature on shoot and root NRA_i



Each point represents the mean of 5 replicates
Error bars mark standard deviations of means


NRA$_i$
(µmol NO_2^-/g fr.wt./h)
0.5
0.4
0.3
0.2
17
25
27
30
31
32
33
34
36
Incubation Temperature
(C)

3.4.8 Rate of NO_2^- accumulation

NR assays *in vivo* can be performed for any given time, and results expressed on a per hour basis, only if NO_2^- is released at a constant rate over the whole assay period (Streeter & Bosler, 1972; Srivastava, 1974 and Lin & Kao, 1980). However, some workers have observed a lag phase of 10-60 minutes, depending on species and technique, before NO_2^- is released at a linear rate (Blahova & Segeta, 1980 and Gebauer *et al.*, 1984). An experiment was therefore performed to determine the rate of NO_2^- release, and to measure the lag phase (if any), for barley (variety Igri), during NR assays *in vivo*.

Leaves and roots from standard plants (section 3.2) were assayed for NRA_e (a NO_3^- free incubation medium was used) and vials were removed from the incubation bath at 20 minute intervals over a 3 hour period for NO_2^- determination.

After an initial lag of about 30 minutes, NO_2^- release from shoots continued in a linear fashion until the end of the experiment (Figure 8). This lag phase may have been due to the time taken for incubation medium to diffuse into shoot tissues and for NO_2^- to diffuse out into the incubation medium.

In view of this 30 minute lag phase, all subsequent shoot samples were incubated for 1 hour and NRA was expressed as the number of micromoles of NO_2^- released within that hour per gram fresh weight of tissue.

In contrast to shoot assays, there was no initial lag phase during root incubations, and NO_2^- was released at its greatest rate during the first 60 minutes of incubation (Figure 9). The absence of a lag phase during root incubations was perhaps due to the fact that roots have no cuticle and will therefore not present as great a

FIGURE 8

Rate of NO_2^- accumulation during the NRA_e assay in vivo of shoots

Results are means of 5 replicates
Error bars mark standard deviations of means

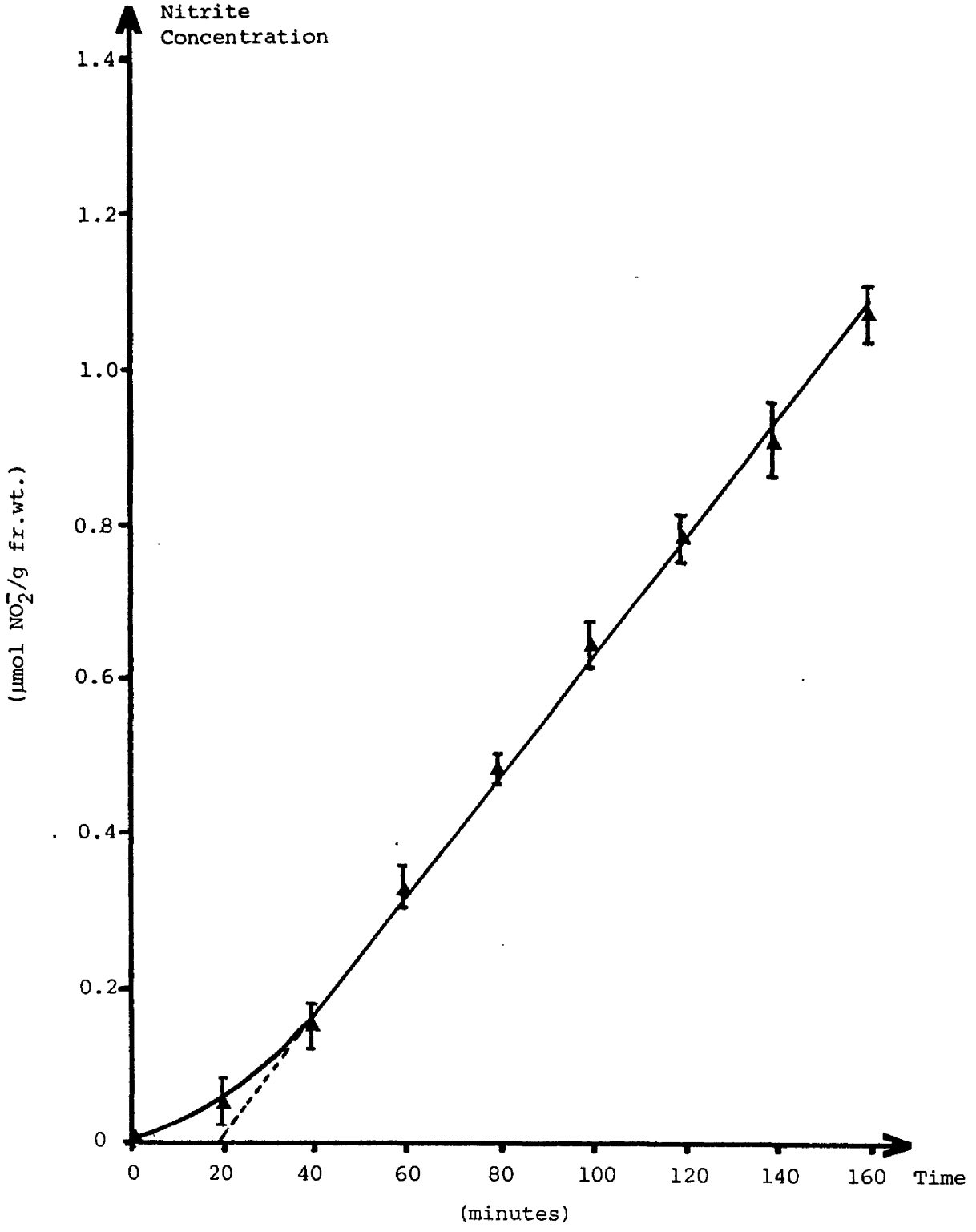
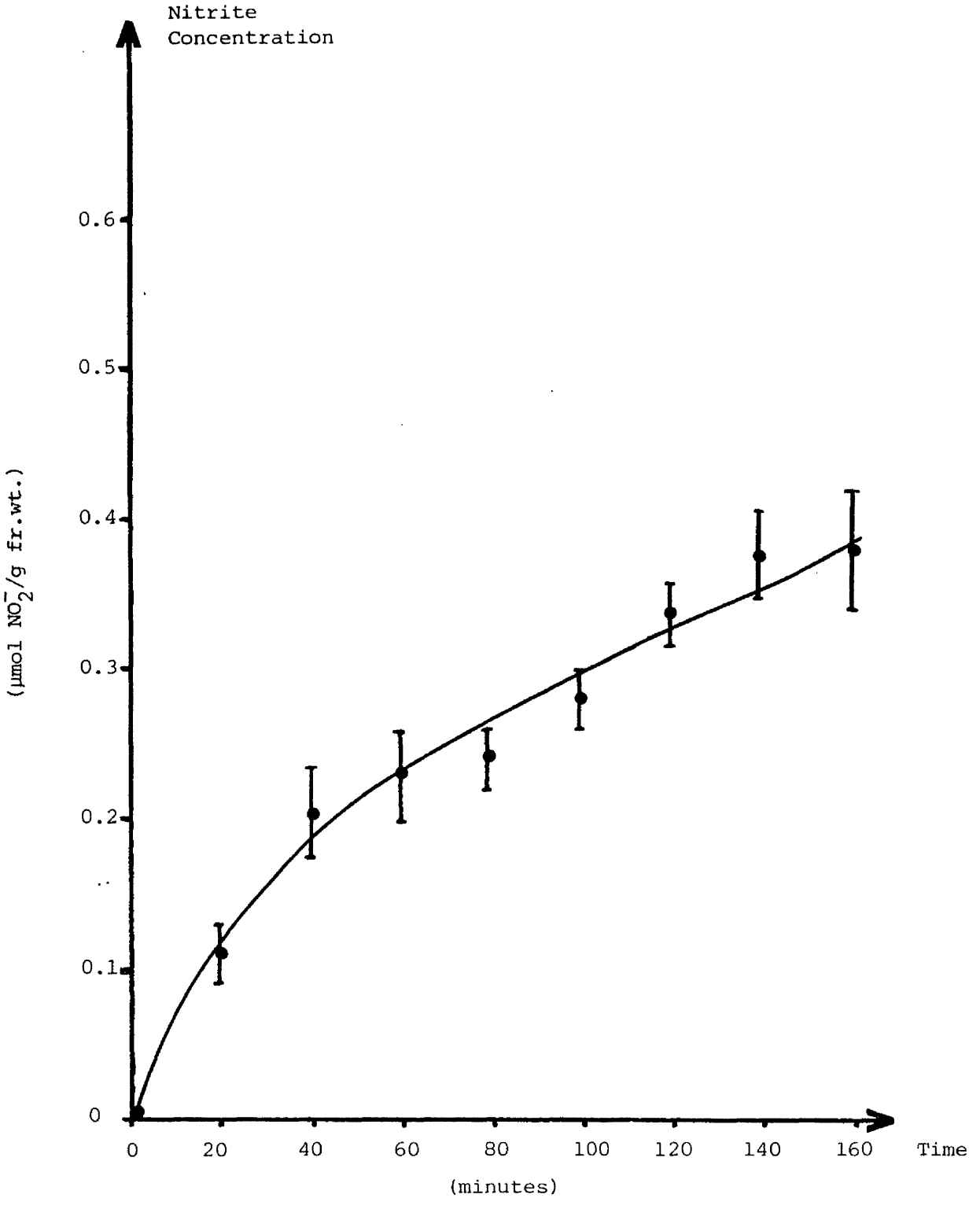

Nitrite
Concentration
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
(µmol NO_2^-/g fr.wt.)
0
20
40
60
80
100
120
140
160
Time
(minutes)

FIGURE 9

Rate of NO_2^- accumulation during the NRA_e assay in vivo of roots

Results are means of 5 replicates
Error bars mark standard deviations of means


Nitrite
Concentration
(µmol NO_2^-/g fr.wt.)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
Time
(minutes)

barrier to diffusion as do leaves.

The decreased rate, and eventual cessation, of NO_2^- production observed during root assays (Figure 9) may have been due to exhaustion of substrate or reductant, or to end-product (NO_2^-) inhibition or inactivation of NR; an effect also observed by Ferrari et al., (1973).

Since NO_2^- release from roots during NR assays was non-linear, it was decided to incubate all root samples for 1 hour and to express NRA as the number of micromoles of NO_2^- released within that hour per gram fresh weight of tissue.

3.4.9 Leaf slice size

The width of leaf slice used in NR assays in vivo affected the amount of NO_2^- produced (Lin & Kao, 1980). There existed an optimal leaf slice size which produced maximum amounts of NO_2^- during NR assays in vivo (Sylvester-Bradley, 1984). This slice size was therefore determined for barley (variety Igri).

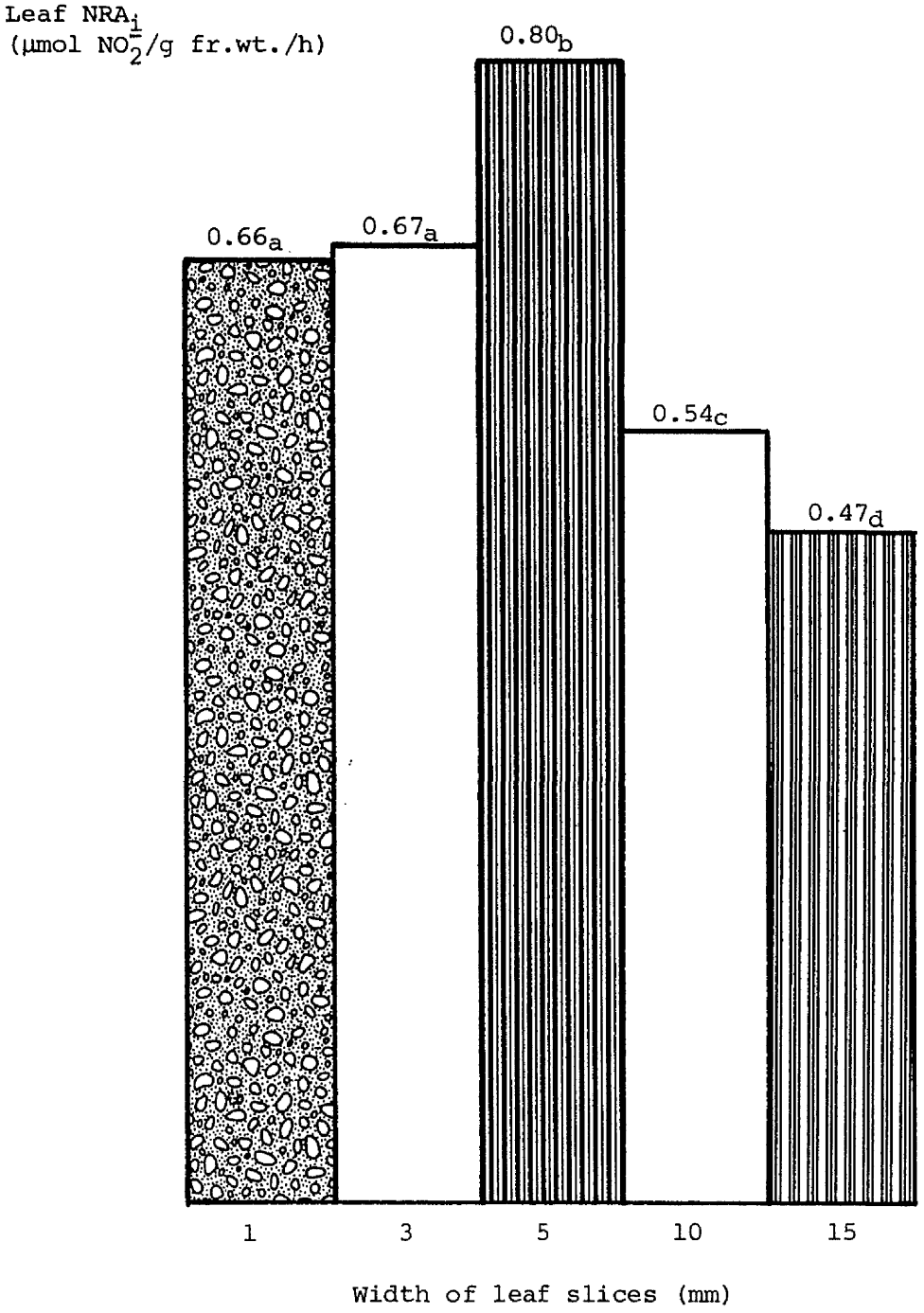
Standard leaf material (section 3.2) was chopped into transverse slices of widths in the range 1-15mm prior to being assayed for NRA_i in incubation medium containing 3% (v/v) propan-1-ol, 0.01% (v/v) Triton X-100, 0.02M KNO_3 and 0.1M phosphate buffer, pH 7.0.

Leaf slices of width 5mm gave highest NRA_i (Figure 10) and were therefore chosen as optimum for subsequent assays. With larger leaf slices, there were fewer cut edges per unit weight of sample through which diffusion could occur, hence reducing the amount of NO_2^- released per unit time compared with smaller leaf slices. When smaller slices were used, many cells would be damaged and inhibitors of NR may have been released, or reductant supply to NR may have been

FIGURE 10

Effect of width of leaf slice used in the assay in vivo on NRA_i –

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level


Leaf NRA$_i$
(µmol NO_2^-/g fr.wt./h)
0.66$_a$
0.67$_a$
0.80$_b$
0.54$_c$
0.47$_d$
1
3
5
10
15
Width of leaf slices (mm)

disrupted, thereby decreasing amounts of NO_2^- produced.

In root assays, samples were chopped into approximately 10mm lengths since it was not practical to precision chop roots to determine optimal root slice sizes.

3.4.10 Miscellaneous factors influencing NO_2^- accumulation

3.4.10.1 Introduction

Many factors other than chemical composition, pH and temperature of the incubation medium may influence NO_2^- accumulation during NR assays *in vivo*, hence affecting assay sensitivity. The following factors were therefore investigated:

1. Pre-incubation
   a) Endogenous NO_2^- (section 3.4.10.2)
   b) Time between sampling and incubation (section 3.4.10.3)
   c) Vacuum infiltration (section 3.4.10.4)
   d) Freeze/thaw treatment (section 3.4.10.5).
2. During incubation
   a) Non-enzymic NO_2^- production (section 3.4.10.6)
   b) Bacterial contamination (section 3.4.10.7)
   c) Anaerobiosis (section 3.4.10.8)
   d) Light (section 3.4.10.9)
   e) NO_2^- reduction (section 3.4.10.10)
   f) Addition of NH_4^+, β-NADH, sugars or L-malate (sections 3.4.10.11 - 3.4.10.14).
3. Post-incubation
   a) Enzyme leakage from tissues (section 3.4.10.15)
   b) Boiling after incubation (section 3.4.10.16).

Optimal NR assay *in vivo* conditions determined earlier (sections 3.4.2-3.4.9) were used throughout these experiments, i.e. material was assayed for NRA_i in incubation medium containing:

	3% (v/v)	propan-1-ol
	0.01% (v/v)	Triton X-100
	0.02M	KNO_3
and	0.1M	phosphate buffer

KNO_3 was omitted from the medium if NRA_e was to be determined. Incubation pH was 7.0 for leaf, and 7.5 for root assays and incubations were performed for 1 hour at a temperature of 32^oC. Leaves were chopped into 5mm wide slices and roots were chopped into approximately 10mm lengths.

3.4.10.2 Endogenous NO_2^-

Any endogenous NO_2^- within plant tissues at the start of NR assays may leak into the medium, be detected after incubation and lead to overestimations of NRA. It was therefore important to measure how much NO_2^-, if any, was present in barley plants prior to NR assays.

Standard leaf and root samples (section 3.2) were harvested at mid-photoperiod, and at the end of the dark period, and heat killed to prevent NR or NiR from producing or consuming NO_2^- respectively. The plant material was then macerated with cold water and samples of filtered solution were analysed for NO_2^-.

No NO_2^- was detected in leaves or roots harvested at mid-photoperiod, although a trace of NO_2^- (< 0.02 μmol NO_2^- g^{-1} fresh weight) was found in shoots of some plants at the end of the dark period. No correction to NRA measurements need therefore be made to

account for these neglectable endogenous NO_2^- levels, as also found by Jones & Sheard (1977).

3.4.10.3 Time between sampling and assay

Although NR assays in vivo are quick and simple, the time taken to chop, mix and weigh a large number of samples can be considerable. It was therefore important to determine how long samples could be left between harvest and assay without significantly altering NRA detected.

Standard barley leaves and roots (section 3.2) were harvested, chopped and bulked separately. Samples were left in the light, at room temperature, and sub-samples were drawn at 10 minute intervals for determination of NRA_e.

TABLE 2: Effect of time between harvest and assay on shoot and root NRA_e.

Time from harvesting (Minutes)	NRA_e (μmol $NO_2^- g^{-1}$ fresh weight h^{-1}) Shoots	Roots
10	0.43_a	0.22_c
20	0.49_b	0.23_c
30	0.52_b	0.29_d
40	0.43_a	0.22_c
50	0.47_{ab}	0.22_c
60	0.54_b	0.19_c

Results are means of 5 replicates.
Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Fluctuations in shoot and root NRA_e were small over the experimental period (Table 2). In subsequent experiments, there was a set period of 60 minutes between harvest and start of incubation to

allow time for sample transportation and preparation. Other workers have stored plant tissues on ice for up to 8 hours without appreciable loss of NRA (Jones et al., 1981).

3.4.10.4 Vacuum infiltration

By application and release of a partial vacuum to plant tissue in incubation medium, the medium is forced into intercellular spaces left by air which is drawn out. It has been shown that vacuum infiltration increases NO_2^- production during NR assays in vivo by up to 40% over control samples, hence increasing assay sensitivity (Aslam, 1981 and Gebauer et al., 1984). This technique was therefore tested for barley (variety Igri).

Samples of standard leaves or roots (section 3.2) were placed in incubation medium (with NO_3^-) and subjected to 3 cycles of application (1 minute) and release (10 seconds) of a partial vacuum. During infiltration, gas bubbled out of leaf tissues and incubation medium, and leaf material rapidly sank. Root tissues sank readily, even before infiltration and no gas was evolved from them during vacuum infiltration. Samples were then incubated as usual, NRA_i values were calculated and compared with those of samples which were not vacuum infiltrated (see Table 3).

TABLE 3: NRA_i of shoots and roots with or without vacuum infiltration prior to NR assay in vivo

	NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	
	Shoot	Root
Not infiltrated	0.70_a	2.0_c
Vacuum infiltrated (3 cycles)	0.96_b	2.0_c

Results are means of 6 replicates.
Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Vacuum infiltration significantly enhanced shoot, but not root, NRA_i. Vacuum infiltration may increase NO_2^- production during leaf NR assays *in vivo* by forcing incubation medium into intercellular air spaces, and bringing the medium into close contact with the leaf cells. Incubation medium entry into roots is likely to be facile, even without vacuum infiltration, since roots do not have as many intercellular air spaces, or a cutin coating, as do leaves.

Vacuum infiltration was not used routinely in subsequent experiments since it involved a much longer preparation time.

3.4.10.5 Freeze/thaw treatment

Freezing of plant tissues in liquid nitrogen, followed by thawing, prior to assaying for NR was shown to cause large increases in NO_2^- production (Ferguson & Sims, 1974 and Rhodes & Stewart, 1974) due to rupture of cell membranes enhancing permeability of cells to diffusion of NO_3^- and NO_2^-. These large increases in activity resulted in enhancement of sensitivity of the NR assay *in vivo*. The freeze/thaw technique was therefore tested for barley (variety Igri).

Standard barley leaves and roots (section 3.2) were placed separately in thin walled tubes and dipped into Dewar flasks of liquid nitrogen for 60 seconds. Samples were then thawed, incubation medium was added and samples were incubated and assayed for NRA_i. Results were then compared with those of control samples which were not frozen (see Table 4).

Freeze/thaw treatment reduced shoot NRA_i to zero and root NRA_i to only 15% of samples which were not frozen. Freeze/thaw treatment was therefore of no use in enhancing NO_2^- production during NR assays *in vivo* of barley and was not used subsequently.

TABLE 4: NRA_i of shoots and roots with or without freeze/thaw treatment prior to assay in vivo

	NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	
	Shoot	Root
Not frozen	0.83_a	0.20_c
Frozen/thawed	0.00_b	0.03_d

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Other workers have also found this technique to cause loss of NRA (Mann et al., 1979; Kadam et al., 1980 and Gebauer et al., 1984). Cold storage of material at only -1^oC greatly reduced NRA (Sylvester-Bradley, 1984). Loss of NRA resulting from freezing may have been due to either disruption of mitochondria, causing loss of production of NADH needed for in vivo NO_3^- reduction (Heber & Santarius, 1964) or to inhibitors or inactivators of NR released when cell membranes were ruptured.

3.4.10.6 Non-enzymic NO_2^- production

NO_2^- detected after NR assays in vivo may not be due solely to the action of NR since there might be some chemical reduction, or NH_4^+ oxidation, during assays. This possibility was therefore investigated by assaying heat killed tissues for NRA.

Standard barley leaves and roots (section 3.2) were heat killed and assayed for NRA_e and NRA_i. No NO_2^- was detected after incubation since heat treatment destroyed the thermolabile NR enzyme, therefore no enzymic NO_3^- reduction took place. Lack of any NO_2^- production shows that no chemical NO_3^- reduction or NH_4^+ oxidation

occurred during incubation. All NO_2^- detected after normal NR assays was therefore probably due to catalytic reduction of NO_3^- by NR, as found by Bar-Akiva & Sternbaum (1965); Bowerman & Goodman (1971) and Srivastava (1974).

Since no NO_2^- was detected after assays of heat killed tissues, this confirms that there were neglectable amounts of endogenous NO_2^- in standard barley leaves or roots, as found previously (section 3.4.10.2).

3.4.10.7 Bacterial contamination

Rhizosphere bacteria may reduce NO_3^- to NO_2^- which could accumulate in the incubation medium during assays, and contribute to overall root NRA detected.

Standard barley leaves and roots (section 3.2) were therefore processed in conjunction with the Microbiology Department to investigate this effect.

Roots had about 10^7 bacteria g^{-1} fresh weight (total viable aerobic count at 25°C) when grown in nutrient solution. Approximately 10% of these bacteria reduced NO_3^- to NO_2^- and, in most samples, NO_2^- was reduced no further and accumulated during a 20 day incubation. Total amounts of NO_2^- produced were small, indicating low rates of bacterial NO_3^- reduction. Due to the small biomass of bacteria and low rates of NO_3^- reduction, bacteria could not have contributed significantly to NO_2^- production during short (1 hour) assays of roots for NRA. NO_2^- detected in root NR assays was therefore essentially all due to the activity of root NR.

Bacteria could not have contributed to NO_2^- production in shoot assays, since a total viable aerobic count revealed only about

100 bacteria g^{-1} fresh weight of shoots.

3.4.10.8 Anaerobiosis

Many workers maximise NO_2^- accumulation during NR assays *in vivo* by using anaerobic conditions, usually achieved by nitrogen flushing during incubation (Klepper *et al.*, 1971; Radin, 1973; Blevins *et al.*, 1976 and Ben-Zioni & Heimer, 1977). However, some workers have demonstrated that nitrogen flushing gives no increase in NO_2^- production during NR assays (Jaworski, 1971 and Brunetti & Hageman, 1976). An experiment was therefore performed to determine whether flushing incubation vials with nitrogen would increase amounts of NO_2^- accumulating during NR assays of barley (variety Igri).

Standard leaves and roots (section 3.2) were assayed for NRA_e in vials flushed with humidified nitrogen gas during the assay. The incubation medium was previously de-aerated by bubbling nitrogen through it. Results were then compared with those of samples incubated as usual in closed air filled vials (see Table 5).

TABLE 5: Effect on NRA_e of assaying shoots and roots under nitrogen gas or in air filled vials

	NRA_e (μmol NO_2^- g^{-1} fresh weight h^{-1})	
	Shoot	Root
Incubated in air filled vials	0.82_a	0.22_b
Incubated under nitrogen	0.81_a	0.20_b

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

No differences in shoot or root NRA_e were detected whether

material was incubated under nitrogen or in air filled vials. Subsequent assays were therefore carried out in closed air filled vials for convenience. Use of propan-1-ol and surfactant in the incubation medium may have caused tissues to "wet" and be waterlogged rapidly, hence making the environment within tissues anaerobic (as thought to be required for maximal NO_2^- accumulation by Jones & Sheard, 1978), without the need for nitrogen flushing.

3.4.10.9 Light during assays

Assays *in vivo* for NRA of leaves must be carried out in darkness to prevent rapid reduction of NO_2^- to NH_4^+, catalysed by NiR (Klepper *et al.*, 1971). When shoot NR assays were performed in the light, some (or all) of the NO_2^- produced by NR was seen to be further reduced (Randall, 1969 and Mann *et al.*, 1979). The effect of light on NO_2^- accumulation during NR assays *in vivo* was therefore investigated.

Standard barley leaves and roots (section 3.2) were assayed for NRA_e and NRA_i in the laboratory light and results were compared with those of normal dark assays (see Table 6).

TABLE 6: Effect of assaying shoots and roots in light or dark on NRA_e and NRA_i

	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
Incubated in light (~ 0.8 klx)	0.02_a	0.31_c	0.46_e	1.09_f
Incubated in darkness	0.37_b	0.90_d	0.50_e	1.19_f

Results are means of 6 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

In shoot NRA_e and NRA_i assays, NO_2^- produced by NR was further reduced in the light. In shoot NRA_e assays, virtually all the NO_2^- produced was reduced in the light, which reflects the situation in growing plants (no NO_2^- was detected in growing plants, section 3.4.10.2). When shoots were incubated in the dark, there was a build-up of NO_2^-, since no Fd_{red} was available as electron donor for NO_2^- reduction. Klepper et al., (1971) and Neyra (1974) have also observed similar results to these.

In root assays, the amounts of NO_2^- produced by NR were similar in light and darkness. NO_2^- was therefore not reduced by NiR at a greater rate in light than in darkness. However, it is possible that some NO_2^- was further reduced during dark assays of shoots and roots (see section 3.4.10.10).

Since leaf NR assays in vivo had to be carried out in darkness to maximise the amount of NO_2^- present after incubation, all leaf (and root) assays were carried out in a darkroom.

3.4.10.10 NO_2^- reduction during dark assays

During NR assays in vivo, dark, partially anaerobic conditions are intended to prevent reduction of NO_2^- to NH_4^+ by NiR. However, some NO_2^- reduction was observed by Srivastava et al., (1980). It was therefore desirable to know how much NO_2^-, if any, was further metabolized during NR assays in vivo of barley, hence decreasing amounts of NO_2^- detected and giving low estimates of NRA.

In preliminary experiments, no NH_4^+ was detected and a spike of exogenously applied NO_2^- was completely recoverable after dark assays. Although NO_2^- reduction was not observed in these experiments, neither result conclusively demonstrated that no NO_2^-

was reduced to glutamine, glutamate or amino acids. There was neither enough time nor facilities available to determine if the amount of NO_3^- reduced was stoichiometric with the amount of NO_2^- accumulated during assays, or if ^{15}N labelled NO_3^- was incorporated into glutamine etc. Either of these methods would have produced quantitative measures of how much NO_2^-, if any, was reduced.

In conclusion, it was not possible to determine the amount of NO_2^-, if any, reduced during dark NR assays *in vivo*. The most important factor, as far as NR assays *in vivo* are concerned, is that NO_2^- reduction takes place at a much slower rate in darkness than in the light. Hence, NO_2^- accumulates during dark NR assays, but not in assays performed in the light or in growing plants in the light (Mann *et al.*, 1979).

3.4.10.11 Ammonium

Addition of NH_4^+ to incubation media during NR assays has been shown to decrease NRA (Smith & Thomson, 1971; Radin, 1973 and Sahulka, 1977), although other workers have found NH_4^+ to increase NRA (Schrader & Hageman, 1967; Mohanty & Fletcher, 1976 and Sihag *et al.*, 1979). Accordingly, the effect on NRA of adding NH_4^+ to NR assays *in vivo* of barley (variety Igri) was investigated.

NH_4Cl, at a final concentration of 30mM, was added to incubation medium prior to assays of standard barley leaves and roots (section 3.2) for NRA_e and NRA_i. Results were then compared with those of samples to which no NH_4^+ was added, and with those of samples to which 30mM KCl was added instead of NH_4Cl (see Table 7).

TABLE 7: Effect on shoot and root NRA_e and NRA_i of adding NH_4Cl or KCl to incubations

	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
No additions (controls)	1.06_a	1.66_c	0.94_e	1.33_g
30mM NH_4Cl added	0.89_b	1.46_d	0.74_f	1.1_h
30mM KCl added	1.04_a	1.71_c	0.94_e	1.37_g

Results are means of 6 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Addition of NH_4Cl to the medium markedly decreased shoot and root NRA_e and NRA_i compared with controls (no additions). Addition of KCl caused no differences in NRA from control values. Decreases in NRA were therefore due to NH_4^+ and not to any osmotic/ ionic strength (salt) effects or to counteranion (Cl^-) effects. NH_4^+ may decrease NRA by acting as an end-product repressor or inhibitor of NR.

3.4.10.12 NADH

Some workers have observed that NADH supply limited NRA during assays and, by adding β-NADH to incubation media, enhancement of NRA could be obtained (Brunetti & Hageman, 1976 and Srivastava et al., 1982). Other workers showed that substrate (NO_3^-) supply limited NRA and that addition of NADH did not increase NRA (Nicholas et al., 1976). An experiment was therefore performed to determine if addition of β-NADH to NR assays in vivo would increase NRA of barley (variety Igri).

β -NADH was added to incubation medium in varying amounts (0.01-0.6mg ml^{-1}) prior to assays of standard leaves and roots (section 3.2) for NRA_e and NRA_i. Samples were subjected to 3 cycles of vacuum infiltration (section 3.4.10.4) prior to incubation to help the β -NADH enter the plant tissues. Samples were then incubated as usual, NRA values were determined and results are shown in Table 8.

TABLE 8: Effect on shoot and root NRA_e and NRA_i of adding β -NADH to incubations

β -NADH concentration (mg ml^{-1} of medium)	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
0 (control)	0.64_a	1.10_b	0.33_d	0.39_e
0.01	0.66_a	1.31_c	0.33_d	0.39_e
0.1	0.67_a	1.31_c	0.33_d	0.41_e
0.6	0.59_a	1.29_c	0.39_d	0.41_e

Results are means of 5 replicates.
Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Addition of β -NADH increased only shoot NRA_i. The lowest concentration added (0.01mg NADH ml^{-1} incubation medium) increased NRA_i over the control value, while larger amounts caused no further increases in shoot NRA_i. The assumption is made that β-NADH actually penetrated the cells, although the large size of the molecule may have hindered its transport across cell membranes (Klepper et al., 1971 and Khan et al., 1984). However, use of vacuum infiltration, propan-1-ol and surfactant should have helped NADH enter the cells.

When NRA was relatively low (in root NRA_e and NRA_i and shoot NRA_e assays), addition of β -NADH caused no increases in NRA,

perhaps indicating that NADH was not limiting NRA. However, when NRA was higher (in shoot NRA_i assays), addition of β-NADH increased NRA, indicating that NADH supply may have been limiting NRA.

3.4.10.13 Sugars

NRA may be coupled to sugar catabolism/carbohydrate metabolism and addition of sugars to assay media caused increases in NRA if plant tissues were deficient in carbohydrates (Sihag et al., 1979 and Puranik & Srivastava, 1983). Accordingly, an experiment was performed to determine if carbohydrate supply was limiting NRA in barley (cultivar Igri).

D(+) glucose or sucrose (2mg ml^{-1} of incubation medium) was added before assays of standard leaves and roots (section 3.2) for NRA_e and NRA_i. Samples were subjected to 3 cycles of vacuum infiltration (section 3.4.10.4) prior to incubation to help the sugars penetrate the tissues. After incubation, NR activities were determined and compared with samples to which no sugars were added (see Table 9).

TABLE 9: Effect on shoot and root NRA_e and NRA_i of adding D(+) glucose or sucrose to incubations

	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
No sugars added	0.83_a	1.10_b	0.52_c	0.76_d
D(+) glucose added (2mg ml^{-1})	0.84_a	1.08_b	0.51_c	0.79_d
Sucrose added (2mg ml^{-1})	0.83_a	1.07_b	0.51_c	0.78_d

Results are means of 5 replicates.
Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

These sugars caused no increases in shoot or root NRA_e or NRA_i at the concentration used, suggesting that carbohydrate supply did not limit NRA. The assumption is made that the use of solvent and surfactant in the incubation medium, and of vacuum infiltration, allowed the sugars to penetrate into the plant cells.

3.4.10.14 L-Malate

Addition of L-malate to incubation media has been found to increase NRA by some workers (Mulder et al., 1959 and Nicholas et al., 1976), but not by others (Srinivasan et al., 1982 and Hipkin et al., 1984). Accordingly, an experiment was performed to determine the effect of L-malate addition to the incubation medium on barley NRA in vivo.

L-malate (at a final concentration of 12mM) was added to incubation medium and vacuum infiltrated (section 3.4.10.4) into shoot and root samples prior to assays for NRA_e and NRA_i. Table 10 shows results for these and for samples to which no L-malate was added.

TABLE 10: Effect on shoot and root NRA_e and NRA_i of adding L-malate to incubations

	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
No L-malate added	1.04_a	1.63_b	0.90_c	1.36_d
12mM L-malate added	1.04_a	1.60_b	0.88_c	1.32_d

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

This concentration of L-malate had no effect on shoot or root NRA_e or NRA_i. The assumption is made that the use of organic solvent, surfactant and vacuum infiltration allowed L-malate to penetrate into the plant cells. Srinivasan et al., (1982) also found that L-malate did not increase NRA.

3.4.10.15 Enzyme leakage from tissues

If active NR (and reductant) leaked out of tissues during NR assays, and was removed in samples for NO_2^- analysis, then NO_2^- production could continue during colour development and give falsely high NRA results.

Occurrence of this was therefore tested for in barley shoot and root samples by removing all plant material from the incubation medium half-way through a one hour assay, analysing the medium for NO_2^- and incubating the remaining medium for a further half hour before determining NO_2^- again.

TABLE 11: Effect of removing shoots or roots from NRA_e assay medium half-way through the assay period on the amount of NO_2^- detected after the assay

	NO_2^- detected (µmol)	
	Shoot	Root
Medium assayed after 30 minutes and all plant tissue removed	0.37_a	0.24_b
Remaining medium incubated for a further 30 minutes after tissue removal	0.36_a	0.22_b

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

No more NO_2^- accumulated in the medium after all the plant material was removed (Table 11). Hence, no active NR and reductant leaked out of shoots or roots. When samples of medium are removed from vials during normal NR assays, NO_2^- production ceases in the aliquot removed and the time of removal is the end of the assay for the purpose of calculating NRA. Other workers have also demonstrated that all reduction takes place within tissues and that no active NR and reductant leaks into the medium (Klepper et al., 1971 and Heuer & Plaut, 1978).

3.4.10.16 Boiling after incubation

It has been suggested that much of the NO_2^- produced during NR assays in vivo is retained within plant tissues and does not diffuse into the medium which is analysed for NO_2^- (Srinivasan et al., 1982). If true, this would lead to an underestimation of NRA and a reduction in assay sensitivity. By boiling previously incubated samples, any retained NO_2^- can be extracted into the medium prior to NO_2^- analysis (Srinivasan et al., 1982). The amount of NO_2^- retained in plant tissues after NR assays in vivo was therefore determined for barley (variety Igri).

Vials containing standard barley leaves or roots (section 3.2) were boiled for 2 minutes after a normal dark incubation (in media with and without NO_3^-), using condensers to prevent concentration of samples by evaporation. Samples were then analysed for NO_2^- and NRA results were compared with samples which were not boiled (see Table 12).

TABLE 12: Effect of boiling samples after incubation on shoot and root NRA_e and NRA_i.

	Shoot		Root	
	NRA_e	NRA_i	NRA_e	NRA_i
	(μmol NO_2^- g^{-1} fresh weight h^{-1})			
Not boiled	1.27_a	1.56_b	0.33_d	0.53_f
Boiled after incubation	1.39_a	1.78_c	0.47_e	0.60_f

Results are means of 5 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Boiling after incubation increased amounts of NO_2^- released, but increases were only statistically significant ($P \leqslant 0.05$) in shoot NRA_i and root NRA_e assays. Hence, most of the NO_2^- produced diffused into the medium during assays. Hog et al., (1983) observed that solution analysis detected about 95% of the NO_2^- produced. Boiling after incubation is therefore not recommended for routine use because of the extra time and equipment necessary.

3.4.11 Conclusions

The optimal incubation medium to maximise NO_2^- production during NR assays in vivo of barley (variety Igri) contained:

3% (v/v) propan-1-ol (section 3.4.2)
0.01% (v/v) Triton X-100 (section 3.4.3)
0.02M KNO_3 (section 3.4.4)
and 0.1M phosphate buffer (section 3.4.5).

Medium of this composition was used routinely to determine NRA_i, while KNO_3 was omitted if NRA_e was being determined. An incubation pH of 7.0 and 7.5 was optimal for leaf and root assays

respectively (section 3.4.6). Optimum incubation temperature was $32^{o}C$ for leaves and roots (section 3.4.7) and all samples were incubated for 1 hour (section 3.4.8). Leaf slices of width 5mm were optimal and approximately 10mm lengths of root material were used (section 3.4.9).

In the study of miscellaneous other factors which could influence NO_2^- accumulation (section 3.4.10), the conclusions reached were:

1. Pre-incubation

   a) There were neglectable amounts of endogenous NO_2^- in growing plants (section 3.4.10.2).

   b) Samples could be left for up to 1 hour between harvest and assay without affecting NRA_e (section 3.4.10.3).

   c) Vacuum infiltrating medium into tissues increased shoot, but not root, NRA_i (section 3.4.10.4).

   d) Freezing plant tissues in liquid nitrogen drastically reduced NRA_i (section 3.4.10.5).

2. During incubation

   a) No non-enzymic NO_2^- production occurred (section 3.4.10.6).

   b) NR of rhizosphere bacteria did not contribute to NO_2^- production (section 3.4.10.7).

   c) Flushing vials with nitrogen gas did not enhance NO_2^- accumulation (section 3.4.10.8).

   d) NO_2^- was rapidly lost by further reduction if incubations of leaf material were performed in the light (section 3.4.10.9).

   e) The amount of NO_2^- (if any) lost by further reduction during dark NR assays could not be quantified with the available resources (section 3.4.10.10).

f) Addition of NH_4^+ caused marked decreases in NRA (section 3.4.10.11).

g) Addition of β-NADH increased shoot NRA_i slightly but had no effect on shoot NRA_e, root NRA_e or root NRA_i (section 3.4.10.12).

h) Addition of sugars or L-malate did not enhance NRA (sections 3.4.10.13 and 3.4.10.14).

3. Post-incubation

a) No active NR leaked out of tissues into samples removed for NO_2^- analysis (section 3.4.10.15).

b) Most of the NO_2^- produced leaked out of the plant tissues, but some additional NO_2^- was released by post-incubation boiling of samples (section 3.4.10.16).

Consideration of these results allowed the development of the optimum assay *in vivo* procedure for NR in barley (variety Igri) (Sym, 1984, Appendix 15). The resulting sensitive, reproducible assay technique was then used routinely in a study of the effects of plant factors and environmental conditions on barley NRA (Chapter 4).

4. Effects of plant factors and environmental conditions on NRA of barley

4.1 Introduction

NRA is influenced by many plant factors and environmental conditions. Accordingly, the effects on barley NRA of some of these factors were investigated and are reported in this chapter under the groupings:

a) Plant factors (section 4.3)

b) Light conditions (section 4.4)

c) Nitrogen nutrition (section 4.5)

d) Controlled environmental conditions (section 4.6)

4.2 Plant Culture

Barley seeds (variety Igri) were sown in perlite contained in 13cm diameter pots. The pots were then watered to saturation with a solution containing ($mg\ l^{-1}$):

P	40	Mg	40	B	0.8	Zn	0.4
K	320	Fe	4	Mo	0.08		
Ca	160	Mn	2	Cu	0.4		

Solution conductivity was 2000 $\mu S\ cm^{-1}$ and pH was 6.3. The pots stood in trays of solution (depth 2.5cm), in a glasshouse (temperature range 15-25^{o}C), shaded from sunlight and supplied with supplemental lighting of average intensity 7 klx for 12 hour photoperiods.

After shoot emergence, plants were thinned to leave 25 seedlings per pot. At this stage, and at subsequent 3 day intervals, the nitrogen free solution was replaced with one containing the above nutrients plus 10mg NO_3^- - N l^{-1}.

The above procedure was used to culture plants for most of the

experiments in sections 4.3-4.5, although plants were subjected to different treatments in later stages of growth, and each pot constituted one replicate. In experiments 4.3.4, 4.3.5 and 4.4.3, plants were cultured as described above, but in a growth cabinet (temperature 20 $\pm$ 1^{o}C, 12 hour photoperiods of intensity 9 klx supplied) instead of the glasshouse.

4.3 Plant factors

4.3.1 Introduction

The effect of the following plant factors on barley NRA were studied:

a) Variety (section 4.3.2)
b) Leaf position (section 4.3.3)
c) 1st leaf age (section 4.3.4)
d) Plant growth stage (section 4.3.5)
e) Part of leaf (section 4.3.6)

4.3.2 Variety and species

Differences in NRA have been observed between plant species (Jones & Sheard, 1977; Radin,1978 and Lillo & Henriksen, 1984) and between varieties of a species (Eck & Hageman, 1974; Dalling et al., 1975; Dalling & Loyn, 1977 and Felix et al., 1981). In contrast, Croy & Hageman (1970) and Dale (1976) noted only small differences in NRA between otherwise very different varieties. NR activities of several barley varieties and one wheat variety were therefore measured to determine if differences in their NRA existed.

The barley varieties 'Midas', 'Golden Promise', 'Piccolo' (spring varieties), 'Igri' and 'Gerbel' (winter varieties) and the

winter wheat (Triticum aestivum L.) variety 'Avalon' were grown as described in section 4.2 until they reached the 4th leaf stage. Shoots of 20 plants of each variety were then harvested and assayed for NRA_e.

'Midas' had a lower, and 'Piccolo' a higher, NRA_e than the other barley varieties and 'Avalon' (Figure 11). These may not be true differences in NRA_e between varieties, since the incubation medium had been optimised for 'Igri', and not for the other varieties. Use of suboptimal medium in the NR assay of these varieties may have resulted in artificially low NRA_e being detected. The barley variety 'Igri' was used as standard in all the other work described in this thesis.

4.3.3 Leaf position

Highest NRA was observed in youngest leaves of plants of several species by Wallace & Pate (1965); Harper & Hageman (1972); Hatam (1978); Blahova & Segeta (1980) and Chatterjee et al., (1980). Other workers have, however, detected highest NRA in the oldest leaves (Kumar et al., 1981 and Smith & James, 1982). Accordingly, an experiment was performed to determine if NRA was highest in older or younger leaves of barley (cultivar Igri).

Plants were grown as described earlier (section 4.2) until they reached the 5th leaf stage when they were harvested. Individual leaves of 20 plants were then chopped separately to provide samples of leaf 1 (oldest) - leaf 5 (youngest) and assayed for NRA_e. Results are presented in Figure 12.

Highest NRA_e was found in older leaves (leaves 1 and 2) even though 1st leaves showed signs of senescence (slight chlorosis, some

FIGURE 11

Shoot NRA_e of various barley varieties and a wheat variety (Avalon)

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level

Shoot NRA_e
(µmol NO_2^-/g fr.wt./h)

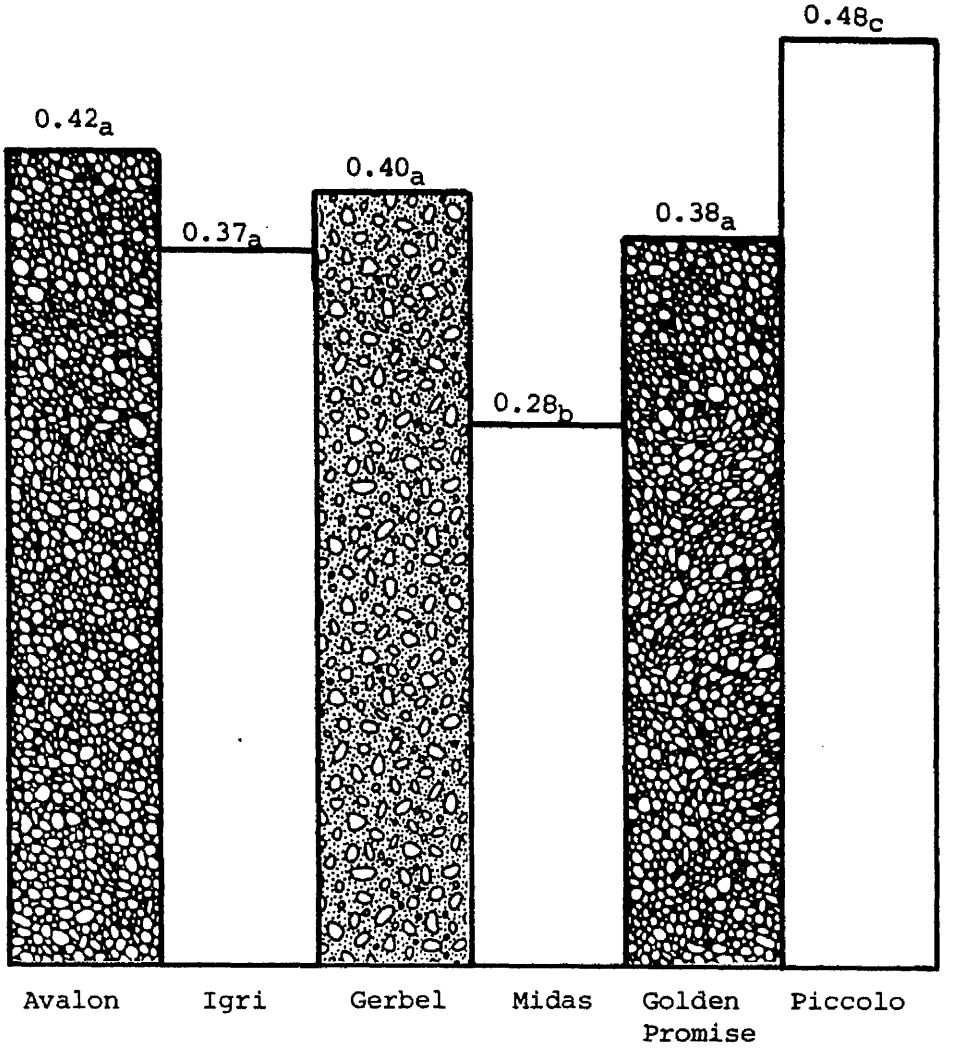
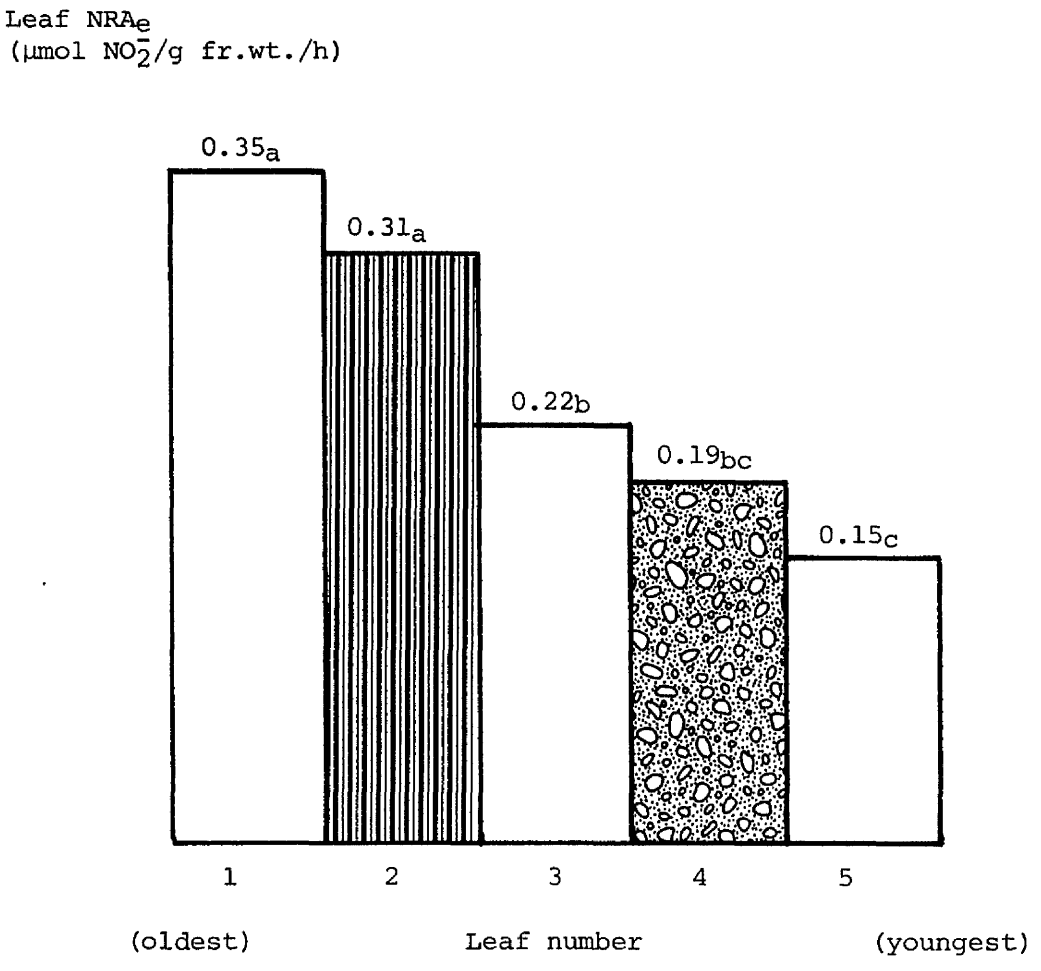


FIGURE 12

Variation in leaf NRA_e with leaf position on the plant

Results are means of 3 replicates
Results followed by the same letter(s) are not
significantly different at the 5% level


Leaf NRA_e
(μmol NO_2^-/g fr.wt./h)
0.35_a
0.31_a
0.22_b
0.19_{bc}
0.15_c
1
2
3
4
5
(oldest)
Leaf number
(youngest)

necrotic spotting). NRA_e was lower in younger leaves (leaves 3 and 4) and lowest in leaf 5, which was not fully expanded.

When results are expressed on a dry weight basis, differences are not so marked due to the greater succulence of younger, than older, leaves. NRA is probably better expressed on a fresh, than on a dry weight basis though, since NR assays *in vivo* are performed on fresh material and measure levels of a metabolically active plant constituent.

4.3.4 NRA_e of 1st leaf as plants age

Blahova and Segeta (1980) observed an increase in NRA to a maximum as the 1st leaf of cucumber plants matured, followed by a gradual decline as the leaf senesced. Franco *et al.*, (1979), using bean plants, also found NRA to vary as leaves aged. In contrast, there was seen to be little decline in NRA as ryegrass leaves senesced (Smith and James, 1982). Accordingly, the variation in NRA_e of 1st leaves of barley plants as they aged was investigated.

Seeds were sown at intervals over 4 weeks and cultured in a growth cabinet as described in section 4.2. Five weeks after the first sowing, the oldest plants had reached the 5th, and the youngest the 2nd leaf stage. The 1st leaves of 20 plants of various ages (7-36 days) were then harvested and assayed for NRA_e.

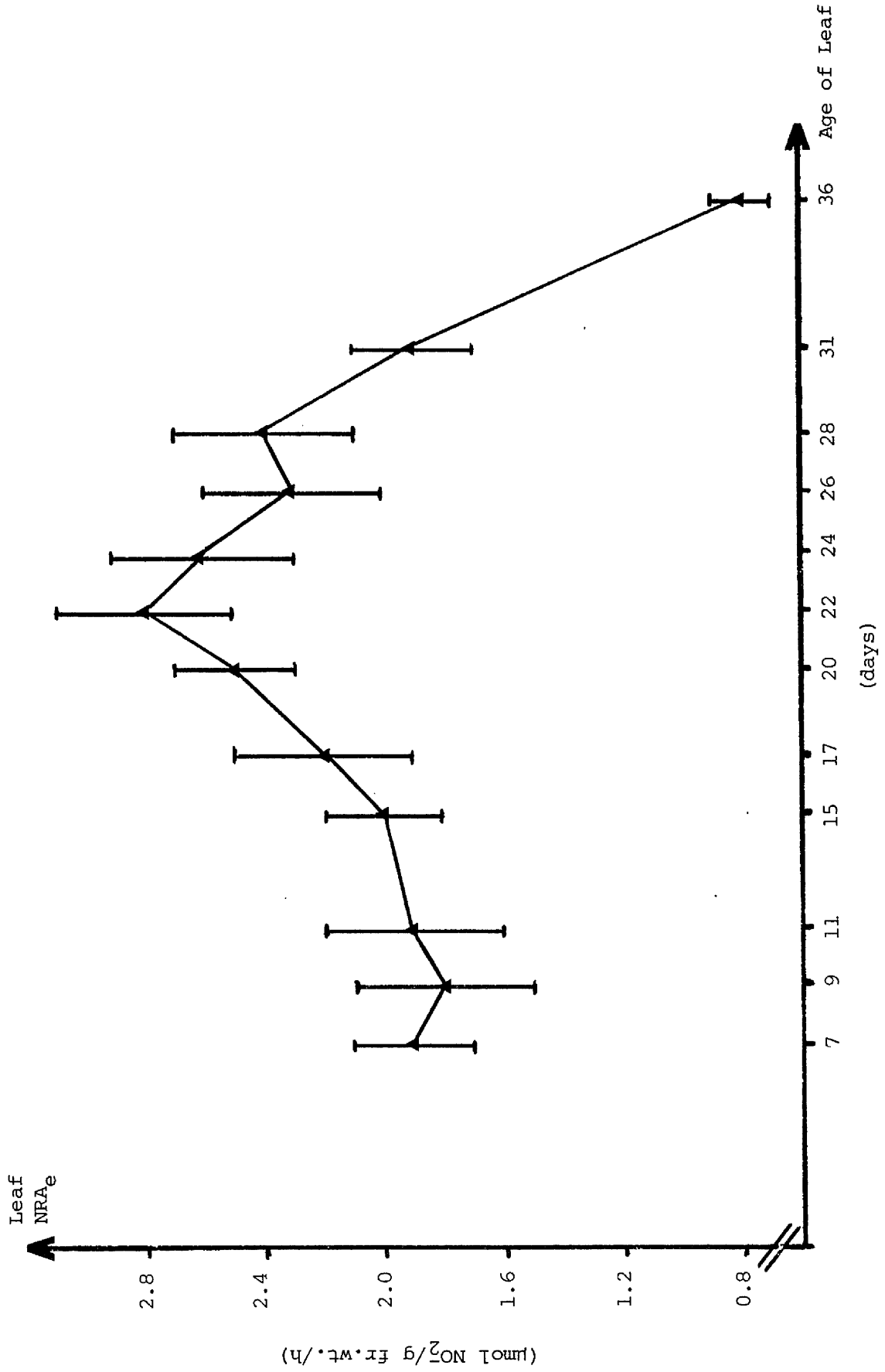
1st leaf NRA_e increased gradually to a maximum 20-28 days after emergence, and then decreased rapidly as the 1st leaf started to senesce (Figure 13). After day 24, the leaf became slightly chlorotic and developed some necrotic spots. Decreased NRA_e as the leaf senesced may have been due to reduction in its protein synthesising capacity or to general protein breakdown as senescence progressed. However, after 36 days, when the 1st leaf was severely chlorotic and

FIGURE 13

Graph of NRA_e versus age of 1st leaf

Each point represents the mean of 3 replicates

Error bars mark standard deviations of means


Leaf
NRA$_e$
2.8
2.4
2.0
1.6
1.2
0.8
(μmol NO_2^-/g fr.wt./h)
7
9
11
15
17
20
22
24
26
28
31
36
Age of Leaf
(days)

necrotic, NRA_e was still one-third of its maximum value.

4.3.5 NRA_e of entire shoots as plants age

Variation in NRA_e of individual leaves as plants age would be a complicating factor if individual leaves were sampled over a period of several weeks to examine effects of, for example, a fertiliser treatment. However, if all the leaves of a plant were to be harvested, bulked and NRA determined, the "entire shoot" NRA might not vary much as plants developed. As each leaf develops, its NRA increases to a maximum and then decreases as time passes (e.g. 1st leaf, section 4.3.4), but peaks of individual leaf NRA will occur at successively later times as further leaves develop, and it is therefore possible that NRA of samples drawn from bulked entire shoots may not vary much as plants develop.

When many treatments were used, the time taken to sample individual leaves was considerable. It was much quicker to harvest entire shoots and determine NRA on samples drawn from the bulked material. Accordingly, NRA_e was determined using entire shoots of barley plants of different ages to determine how "entire shoot" NRA_e varied over a period of a few weeks.

Plants were raised in a growth cabinet as described in sections 4.2 and 4.3.4 to provide material of different ages. Entire shoots of 20 plants of various ages (14-35 days), were then harvested, bulked separately, chopped and assayed for NRA_e.

There were no marked differences in entire shoot NRA_e of plants between the 2nd, and 5th, leaf stages (Table 13). Since entire shoot NRA_e did not vary between these growth stages, any complicating effects of variations in NRA_e with individual leaf age were avoided by sampling entire shoots. Entire shoot sampling was therefore used subsequently for this reason and for speed.

TABLE 13: Effect of plant growth stage on entire shoot NRA_e

Plant growth stage (Zadoks)	Plant age (days)	Entire Shoot NRA_e ($\mu mol\ NO_2^-\ g^{-1}$ fresh weight h^{-1})
12	14	0.42_{ab}
13	18	0.45_{ab}
13	21	0.42_{ab}
14	24	0.44_{ab}
14	27	0.41_{a}
14	30	0.46_{ab}
15	33	0.47_{b}
15	35	0.43_{ab}

Results are means of 5 replicates.

Results followed by the same letter(s) are not significantly different at the 5% level.

4.3.6 Part of leaf assayed

As well as varying with leaf position (section 4.3.3), and leaf age (section 4.3.4), NRA was found to vary with the particular portion of an individual leaf assayed. Highest NRA was detected in the distal portion of cereal leaves by Jordan & Huffaker (1972) and Hallam and Blackwood (1979), but Mack et al., (1984) discovered NRA to be highest in proximal tissues. Accordingly, the variation in NRA_e along the length of the 2nd leaf of barley plants was studied.

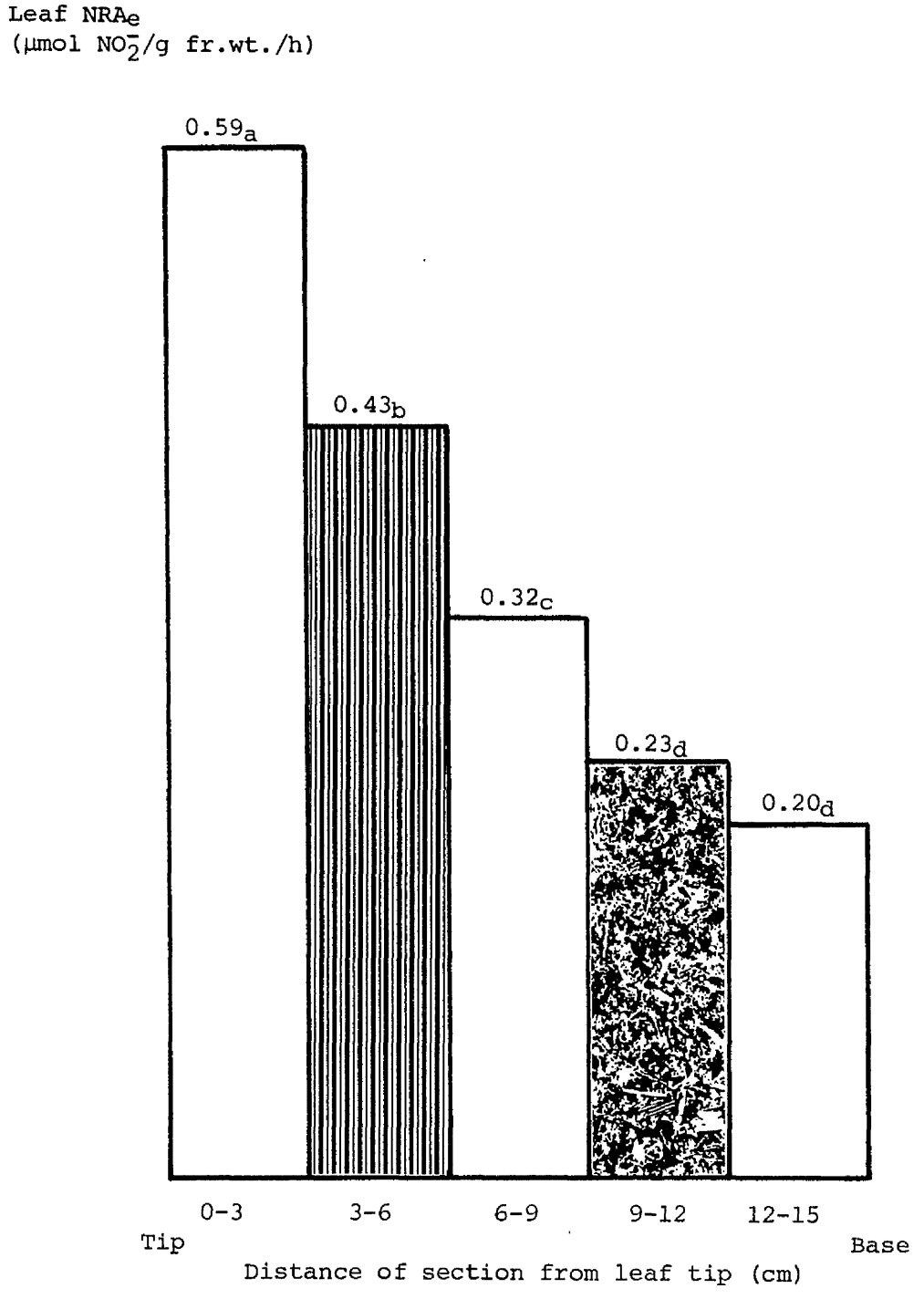
Plants were grown as described before (section 4.2) until they reached the 5th leaf stage. The 2nd leaves of 25 plants were then harvested, cut into 3cm lengths, separated according to distance from the distal tip and assayed for NRA_e.

Highest NRA_e was found in distal tissues with successively lower NRA_e being detected in sections further from the leaf tip (Figure 14). Distal tissues are the oldest parts of the leaf, since

FIGURE 14

Variation in NRA_e with distance from tip of the 2nd leaf of barley

Results are means of 5 replicates
Results followed by the same letter(s) are not significantly different at the 5% level


Leaf NRAe
(µmol NO_2^-/g fr.wt./h)
0.59a
0.43b
0.32c
0.23d
0.20d
0-3
3-6
6-9
9-12
12-15
Tip
Base
Distance of section from leaf tip (cm)

monocotyledonous cereal leaves grow by a basal intercalary meristem. Proximal tissues are the youngest parts of the leaf and may not have been fully mature.

NRA_e may, in part have been higher in leaf tips because there were more leaf fragments in a 500mg sample of narrow, pointed leaf tips than in samples taken further from the tip. There were therefore more cut edges through which diffusion of NO_3^- and NO_2^- could take place in leaf tip samples, and so rates of NO_2^- production might have been greater. This effect becomes much less in samples taken further from the leaf tip, and variations in NRA_e observed along the rest of the leaf length were probably due to different stages of tissue maturity or substrate (NO_3^-) availability.

4.3.7 Conclusions

The following conclusions were reached in the study of the effects of plant factors on NRA of barley:

a) Actual differences in NRA_e between varieties and species could not be quantified (section 4.3.2)

b) NRA_e was highest in the oldest (bottom) leaves (section 4.3.3)

c) NRA_e of 1st leaves increased gradually to a maximum and then declined rapidly as the leaves senesced (section 4.3.4)

d) Complicating effects of the age dependent NRA_e of individual leaves were avoided when entire shoots were sampled and assayed for NRA_e (section 4.3.5)

e) NRA_e of the 2nd leaf was highest in the oldest tissues at the distal tip (section 4.3.6).

4.4 Light and dark

4.4.1 Introduction

Photosynthesis indirectly supplies reductant (NADH) for NO_3^- reduction, and photosynthates for incorporation with reduced nitrogen to form amino acids and proteins, and therefore light (intensity and duration) has a marked effect on NRA.

The effect of the following factors on barley NRA were therefore investigated:

a) Light intensity (section 4.4.2)

b) Diurnal variation (section 4.4.3)

c) Darkness (section 4.4.4)

d) Light after a long dark period (section 4.4.5)

4.4.2 Light intensity

The light intensity under which plants were grown affected NO_3^- uptake rates and NRA (Beevers & Hageman, 1972 and Nicholas et al., 1976). A linear decline in maize NRA as light intensity decreased was observed by Hageman (1960), but other workers have, however, found about 15% of the maximum light intensity used to support as much as 50% of the maximal NRA (Bowerman & Goodman, 1971 and Jones & Sheard, 1977). The relationship between light intensity under which barley plants were grown and shoot NRA_e and NRA_i was therefore studied.

Plants were grown as described in section 4.2 until they reached the 2nd leaf stage and then various light intensities (up to a maximum of 6.6 klx) were imposed. Plants were grown until those least shaded reached the 4th leaf stage. During this time, the most heavily shaded plants did not develop beyond the 2nd leaf stage.

Shoots from all the treatments were then harvested and assayed for NRA_e and NRA_i.

Figure 15 illustrates shoot NRA_e and NRA_i versus % of maximum light intensity (6.6 klx) under which plants were grown. About 50% of the maximum light intensity used sustained 90% of maximal NRA_e and NRA_i, while about 10% of the maximum intensity used sustained 50% of maximal NRA_e and NRA_i.

Figure 16 shows a plot of log (% of maximum light intensity used) versus NRA_e and NRA_i, which suggests that there was a log-linear relationship between light intensity and barley shoot NRA_e and NRA_i. A similar relationship was observed for ryegrass by Smith & James (1982).

4.4.3 Diurnal variation

Many workers have observed diurnal variations in leaf NRA of many plant species (Bowerman & Goodman, 1971; Harper & Hageman, 1972; Bilal & Rains, 1973 and Robin, 1979). Some workers have, however, detected little diurnal variation in leaf NRA (Smith & James, 1982 and Harris & Whittington, 1983). Accordingly, barley shoot NRA_e was monitored over a period of several hours to determine if a diurnal variation in activity existed.

Plants were grown in a growth cabinet, under lights of fixed intensity, as described previously (section 4.2) until they reached the 3rd leaf stage. Shoots of 25 plants were then harvested at hourly intervals during a 12 hour photoperiod and assayed for NRA_e.

NRA_e increased as time passed, to a maximum 8 hours after the start of the photoperiod, and then decreased in the period 9-10 hours after the start of the photoperiod (Figure 17).

FIGURE 15

Graph of shoot NRA_e and NRA_i versus % of maximum light intensity under which plants were grown

Shoot NRA_e △——△

Shoot NRA_i ▲——▲

Each point represents the mean of 4 replicates

Error bars mark standard deviations of means

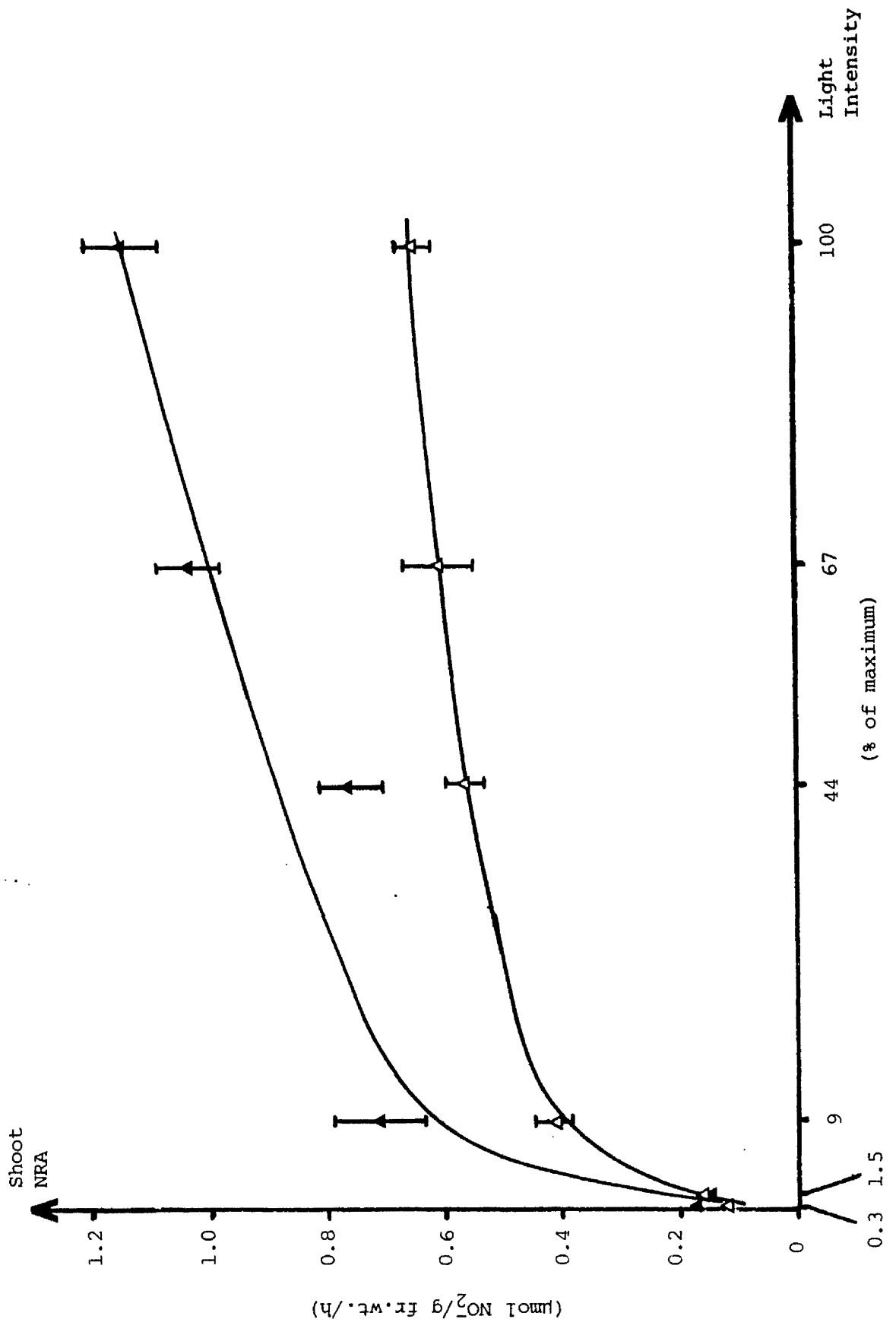

Shoot NRA
(μmol NO_2^-/g fr.wt./h)
1.2
1.0
0.8
0.6
0.4
0.2
0
0.3
1.5
9
44
67
100
Light Intensity
(% of maximum)

FIGURE 16

Graph of log (% of maximum light intensity used) versus shoot NRA_e and NRA_i.

Shoot NRA_e △——△

Shoot NRA_i ▲——▲

Each point represents the mean of 4 replicates

Error bars mark standard deviations of means

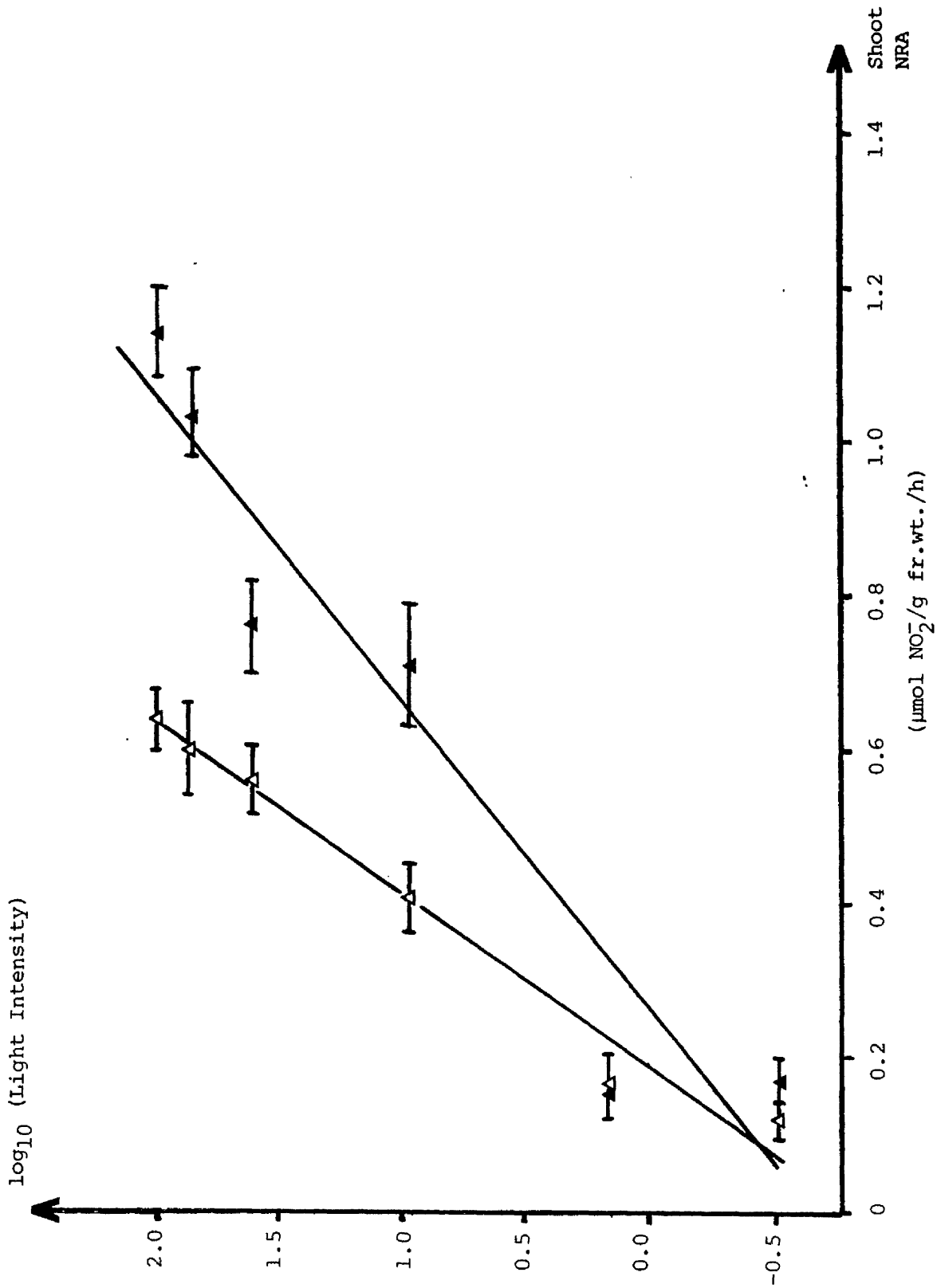
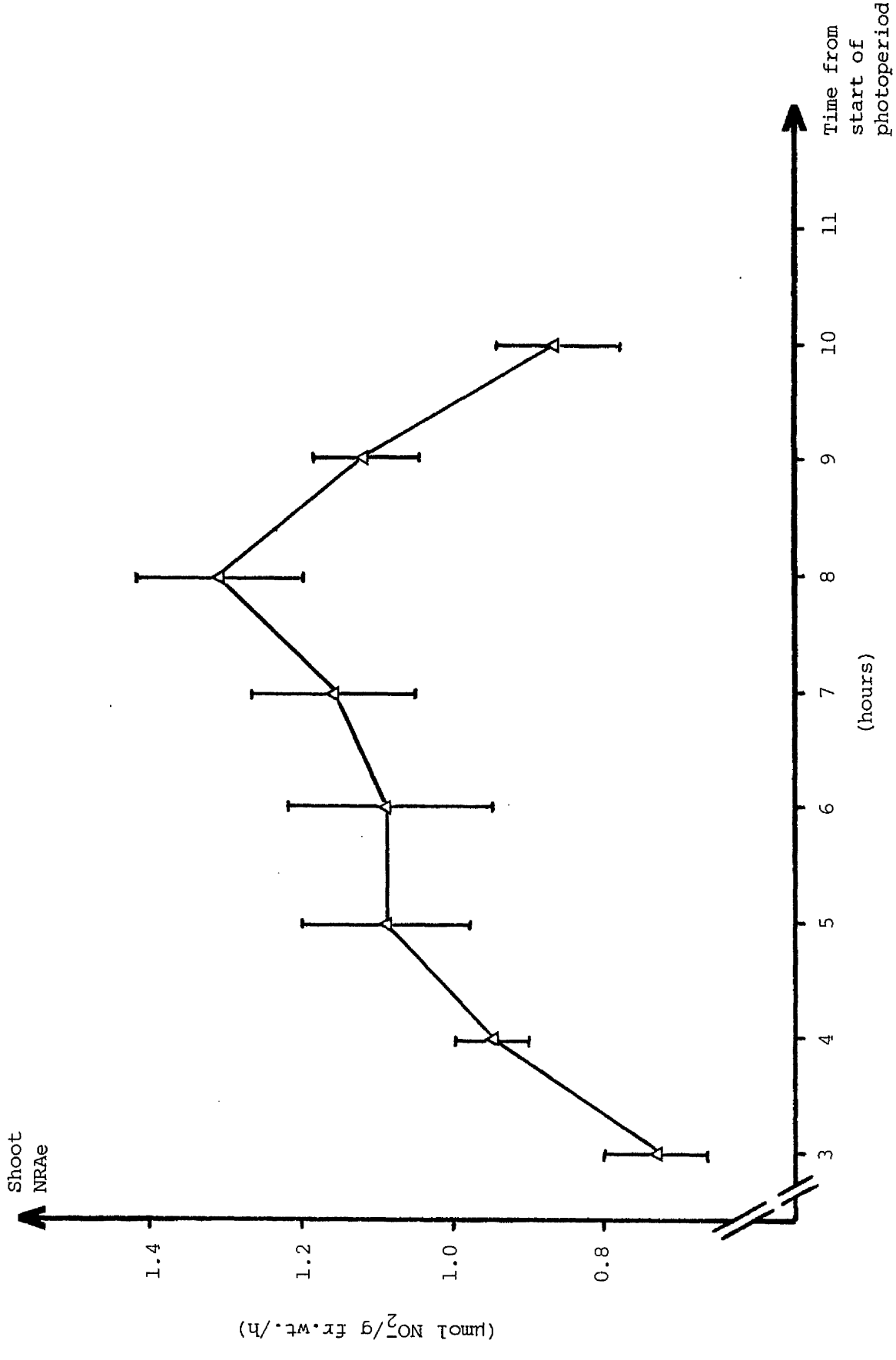

log10 (Light Intensity)
2.0
1.5
1.0
0.5
0.0
-0.5
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Shoot NRA
(µmol NO2-/g fr.wt./h)

FIGURE 17

Graph of shoot NRA_e versus time after the start of the photoperiod

Each point represents the mean of 5 replicates

Error bars mark standard deviations of means


Shoot NRAe
(μmol NO_2^-/g fr.wt./h)
1.4
1.2
1.0
0.8
3
4
5
6
7
8
9
10
11
(hours)
Time from start of photoperiod

Increases in NRA during the first 8 hours of the photoperiod may have been due to light increasing the supply of substrate (NO_3^-) or reductant (NADH) to NR, as proposed by Kannangara & Woolhouse (1967) and Bowerman & Goodman (1971). Decreases in NRA later in the photoperiod might have been due to lack of substrate or reductant or even to NO_2^- accumulating in sufficient quantities at intracellular sites to cause end-product inhibition of NR (although NO_2^- was not detected in growing plants, see section 3.4.10.2).

4.4.4 Darkness

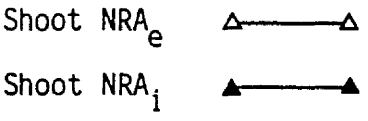
When plants were placed in darkness, photosynthesis stopped and the resulting lack of carbohydrates or reductant caused NRA to decrease (Beevers & Hageman, 1969 and Aslam, 1979). Alternatively, inhibitors of NR may have accumulated in the dark (Travis et al., 1969). Conditions in the dark will not be conducive to net synthesis of new NR protein, and eventually all the original NR will be inactivated or degraded. Accordingly, the rate of decline of barley NRA in the dark was determined.

Plants were grown as described earlier (section 4.2) until they reached the 3rd leaf stage. Shoot and root samples were taken immediately prior to the plants being placed in the dark at a temperature of 20°C. Subsequent sampling took place at 24 hour intervals and all samples were assayed for NRA_e and NRA_i. Fresh nutrients were supplied each day to avoid any confounding effects due to nitrogen depletion.

Shoot NRA_e declined by 50% after only 24 hours in darkness, while NRA_i declined by about 25% during the same period (Figure 18). Shoot NRA_e and NRA_i then declined to low levels after 4 days of

FIGURE 18

Graph of shoot NRA_e and NRA_i versus time plants were kept in darkness



Each point represents the mean of 6 replicates
Error bars mark standard deviations of means

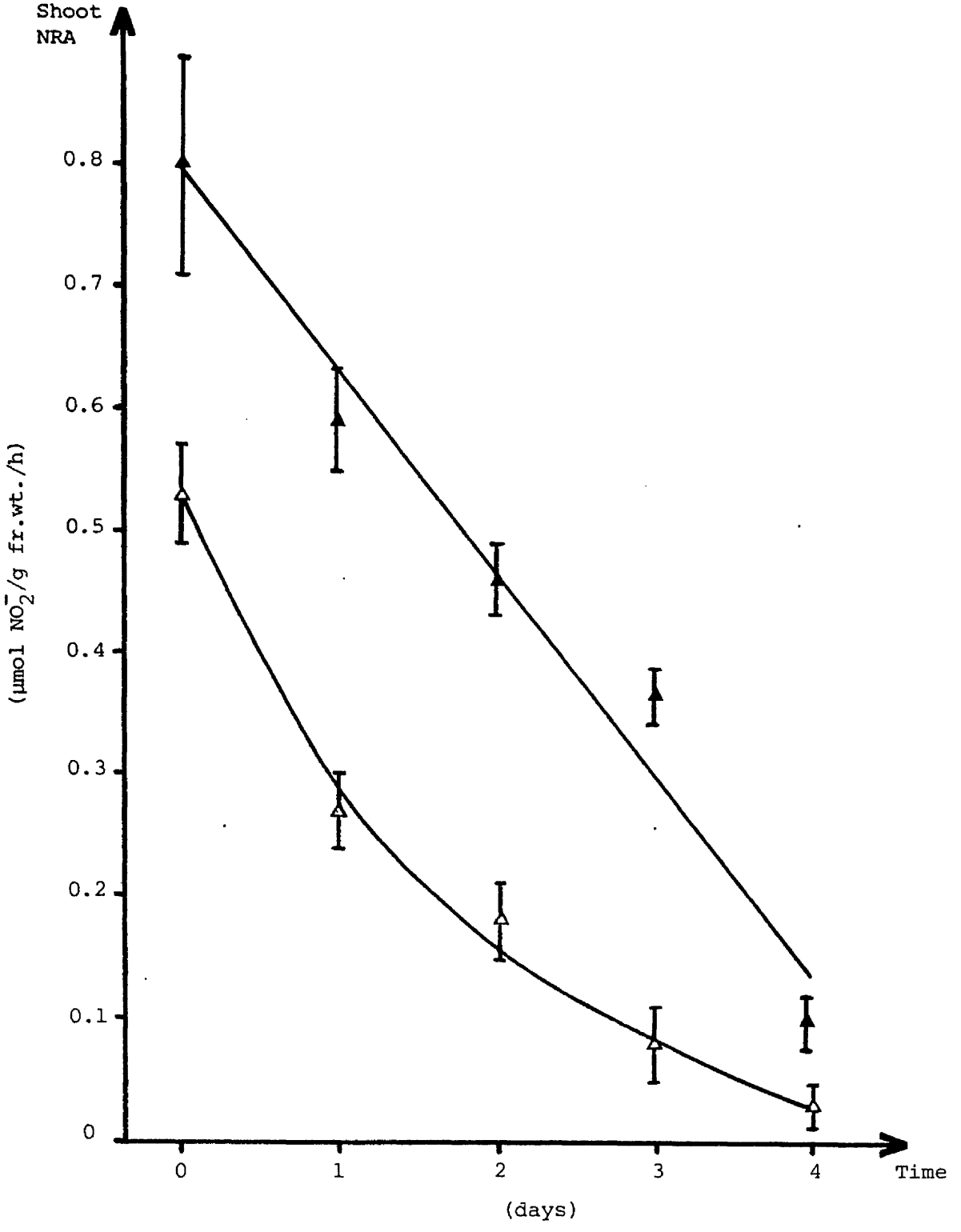
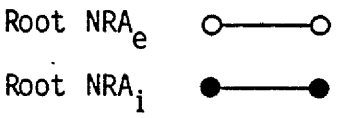
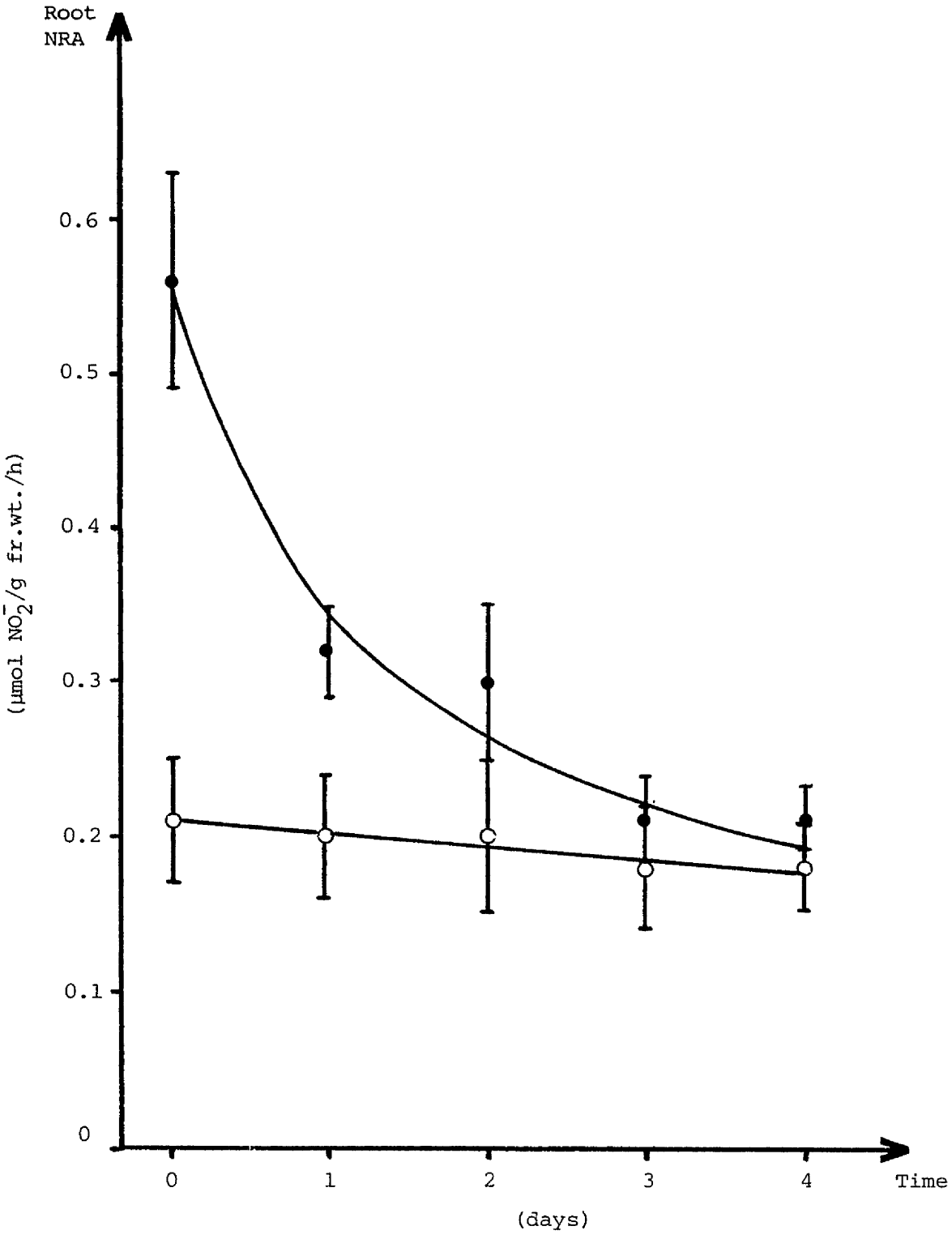

Shoot
NRA
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
3
4
Time
(days)

FIGURE 19

Graph of root NRA_e and NRA_i versus time plants were kept in darkness



Each point represents the mean of 6 replicates
Error bars mark standard deviations of means


Root
NRA
0.6
0.5
0.4
0.3
0.2
0.1
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
3
4
Time
(days)

darkness. Hageman et al., (1960) and Travis et al., (1969) also found NRA of maize and barley to decrease by about 50% after 24 hours in darkness and proposed that loss of NRA under such conditions depended on de novo protein synthesis, since loss was suppressed by inhibitors of protein synthesis.

In contrast to shoot NRA_e, root NRA_e showed no marked decline even after 4 days in darkness, although root NRA_i declined to the level of root NRA_e 3 days after placing plants in darkness (Figure 19).

4.4.5 Light after a long dark period

After a long period in darkness, barley NRA decreased to low values (section 4.4.4) due to lack of reductant or to NR inactivation or degradation. When dark-treated plants are returned to the light and photosynthesis recommences, the rate of increase in NRA will be governed by the rate of NR synthesis and reductant availability. Accordingly, the rate of recovery of NRA when barley plants were returned to the light after a long period in darkness was investigated.

Plants were grown as described previously (section 4.2) until they reached the 3rd leaf stage. They were then placed in darkness for 6 days at a temperature of 20°C and fresh nutrients were supplied every 2 days. Plants were then returned to the light after this dark period and shoots and roots were assayed for NRA_e and NRA_i immediately and at subsequent 24 hour intervals.

At the end of the dark period, the leaves were very chlorotic and there were some necrotic spots on older leaves. No shoot NRA_e was found at this time, although a low NRA_i was detectable (Figure 20), and root NRA_e and NRA_i were both low (Figure 21). Shoot NRA_e and NRA_i were readily detected after 1 day in the light. After 2 days,

FIGURE 20

Graph of shoot NRA_e and NRA_i versus time after returning plants to the light

Shoot NRA_e △——△

Shoot NRA_i ▲——▲

Each point represents the mean of 5 replicates
Error bars mark standard deviations of means

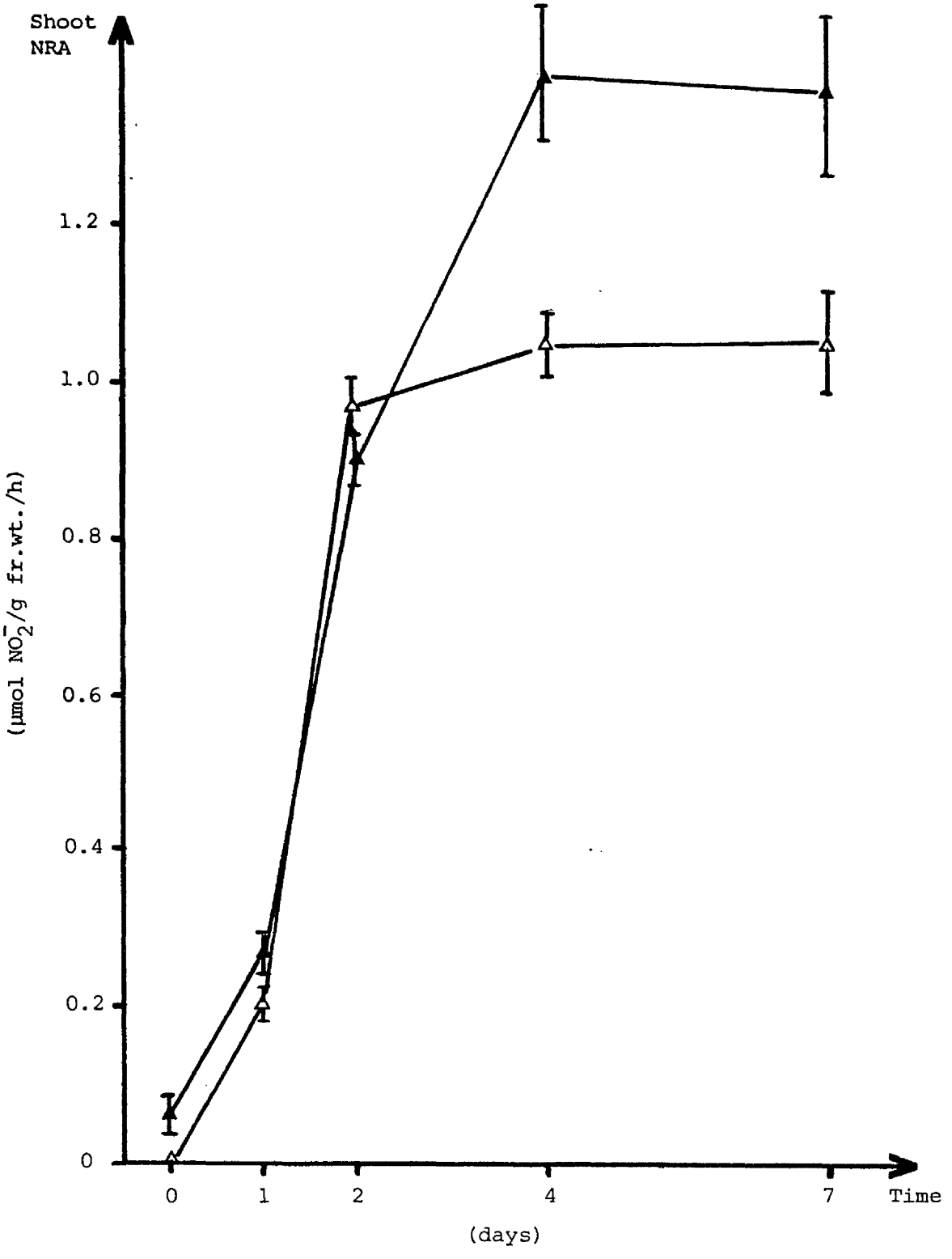

Shoot
NRA
1.2
1.0
0.8
0.6
0.4
0.2
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
4
7
Time
(days)

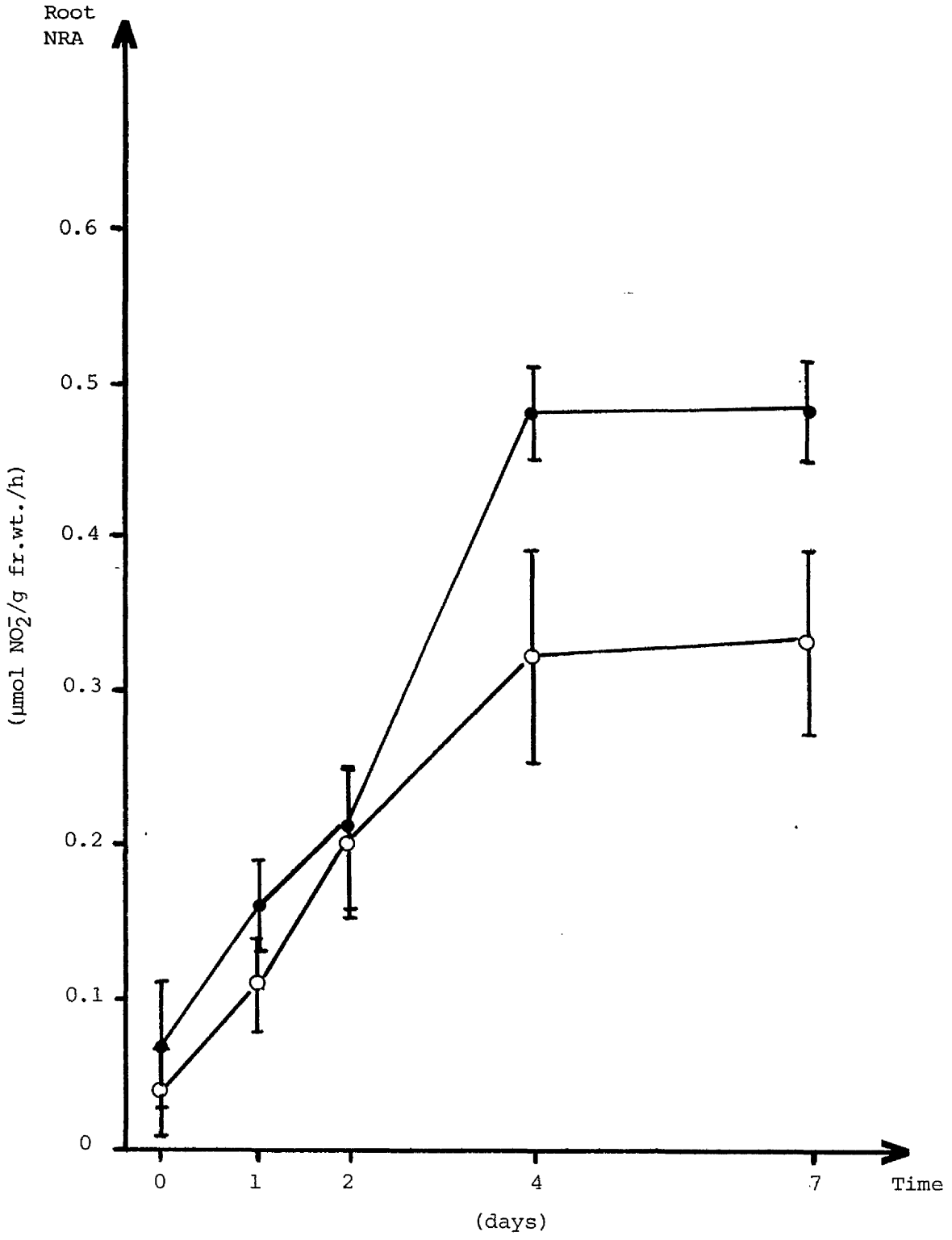
FIGURE 21

Graph of root NRA_e and NRA_i versus time after returning plants to the light

Root NRA_e o——o

Root NRA_i ●——●

Each point represents the mean of 5 replicates
Error bars mark standard deviations of means


Root
NRA
0.6
0.5
0.4
0.3
0.2
0.1
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
4
7
Time
(days)

relatively high shoot NRA_e and NRA_i levels were observed, while maximum plateau levels of shoot and root NRA_e and NRA_i were reached within 4 days of returning plants to the light. The chlorotic symptoms of the leaves largely disappeared during this time, but there remained many necrotic spots, especially on older leaves.

Sawhney & Naik (1972) noted that NR synthesis started within 3 hours of returning etiolated rice seedlings to the light, while Travis et al., (1969) observed a 6-hour lag before barley NRA began to increase, with maximal NRA occurring 24-48 hours after returning plants to the light.

4.4.6 Conclusions

The following conclusions were reached in the study of effects of light conditions on barley NRA:

a) There was a log-linear type relationship between light intensity and shoot NRA_e and NRA_i (section 4.4.2)

b) Shoot NRA_e exhibited a marked diurnal variation (section 4.4.3)

c) When plants were placed in darkness, shoot NRA_e and NRA_i declined to very low values within 4 days. Root NRA_e did not decline much during this period, but root NRA_i declined to the level of root NRA_e within 3 days after placing plants in darkness (section 4.4.4)

d) Shoot and root NRA_e and NRA_i recovered from very low levels to reach maximum, plateau values 4 days after returning plants to the light after 6 days of darkness (section 4.4.5).

4.5 Nitrogen nutrition

4.5.1 Introduction

Since NR is a substrate inducible enzyme, nitrogen nutrition will have an influence on NRA. Accordingly, the effects of the following factors on barley NRA were studied:

a) NRA in the absence of NO_3^- (section 4.5.2)

b) Induction by NO_3^- (section 4.5.3)

c) Cessation of NO_3^- supply (section 4.5.4)

d) NH_4NO_3 nutrition (section 4.5.5)

e) Field observations (section 4.5.6).

4.5.2 NRA in the absence of NO_3^-

NR is a substrate inducible enzyme and, as such, its activity is positively related to NO_3^- availability (Beevers & Hageman, 1969; Wray & Filner, 1970 and Aslam et al., 1973). In addition, NO_3^- has been demonstrated to be essential for the induction and maintenance of NRA (Schrader et al., 1968 and Zeilke et al., 1971). Hence, in plants fed no NO_3^-, NRA is usually low (Shen, 1969). It was desirable to determine the NRA of plants grown without NO_3^- for comparison with levels of activity in plants supplied with NO_3^-. Accordingly, the "background" NRA of barley plants grown without NO_3^- was determined.

Plants were grown until they reached the 3rd leaf stage, as described in section 4.2, but without NO_3^- in the nutrient solution. They were then harvested at the middle of the photoperiod and shoots and roots were assayed for NRA_e and NRA_i. Results are presented in Table 14.

TABLE 14: Shoot and root NRA_e and NRA_i of plants grown without NO_3^-

	NRA_e (μmol NO_2^- g^{-1}	NRA_i fresh weight h^{-1})
Shoots	0.00	0.03 $\pm$ 0.02
Roots	0.00	0.13 $\pm$ 0.03

Results are means of 12 replicates.

NRA_e was not detected in shoots or roots, which confirms the findings of Afridi & Hewitt (1964), Aslam (1982) and Somers (1983). After a 1 hour incubation with NO_3^-, very low levels of NRA_i were found, suggesting substrate (NO_3^-) induction of NR in excised plant material even during the short incubation period.

4.5.3 Induction by NO_3^-

Substrate induction of NR was demonstrated in excised plant material which had been grown without NO_3^- (see section 4.5.2). In the following two experiments, the rate of induction of NR in intact barley plants was studied after supplying NO_3^- to plants previously grown without NO_3^-. In the first experiment, shoot NRA was measured over a period of several hours after first supplying NO_3^-, while in the second experiment, shoot and root NRA were monitored over a period of several days after first supplying NO_3^-.

Plants were grown as described in section 4.2 until they reached the 3rd leaf stage, but NO_3^- was not included in the otherwise complete nutrient solution. A complete nutrient solution containing 10mg NO_3^- - $N\,l^{-1}$ was then supplied to the plants 4 hours after the start of the photoperiod. Shoot samples were taken at hourly intervals and assayed for NRA_e and NRA_i.

No shoot NRA_e was detected at time zero (confirming previous findings, see section 4.5.2), but trace levels were detected within 3 hours after first supplying NO_3^- (Figure 22). Shoot NRA_i increased more rapidly than shoot NRA_e with low levels being found only 1 hour after supplying NO_3^-.

Thus, rapid appearance of shoot NRA indicated fast NO_3^- uptake and transport followed by activation or de novo synthesis of NR, as shown by Afridi & Hewitt (1962), Wallace & Pate (1965) and Dale et al., (1974).

In the second experiment, shoot and root samples were assayed for NRA_e and NRA_i at 24 hour intervals after supplying NO_3^- to plants previously grown in nitrogen-free nutrient solution.

Shoot and root NRA_e and NRA_i increased rapidly (Figures 23 and 24) to maximum, plateau levels 48 hours after first supplying NO_3^-. This compares to a period of 24-72 hours observed by other workers (Brunetti & Hageman, 1976; Somers et al., 1983 and Kaplan & Lips, 1984).

Plateau levels of NRA corresponded to maximal substrate induction of NR under the prevailing conditions. NO_3^- supply should not have been limiting, since nutrients were replenished daily. Further increases in NRA were therefore prevented by some other limiting factor (e.g. reductant supply).

4.5.4 Cessation of NO_3^- nutrition

The presence of NO_3^- in nutrient solution, and a continuous flux to the leaves, has been shown to be necessary to induce and maintain high NRA (Shaner & Boyer, 1976). When NO_3^- supply is cut off, there is a lack of substrate available to induce activation or de novo

FIGURE 22

Shoot NRA_e and NRA_i versus time (hours) after supplying NO_3^- to plants previously grown without NO_3^-

Shoot NRA_e △——△

Shoot NRA_i ▲——▲

Each point represents the mean of 5 replicates

Error bars mark standard deviations of means

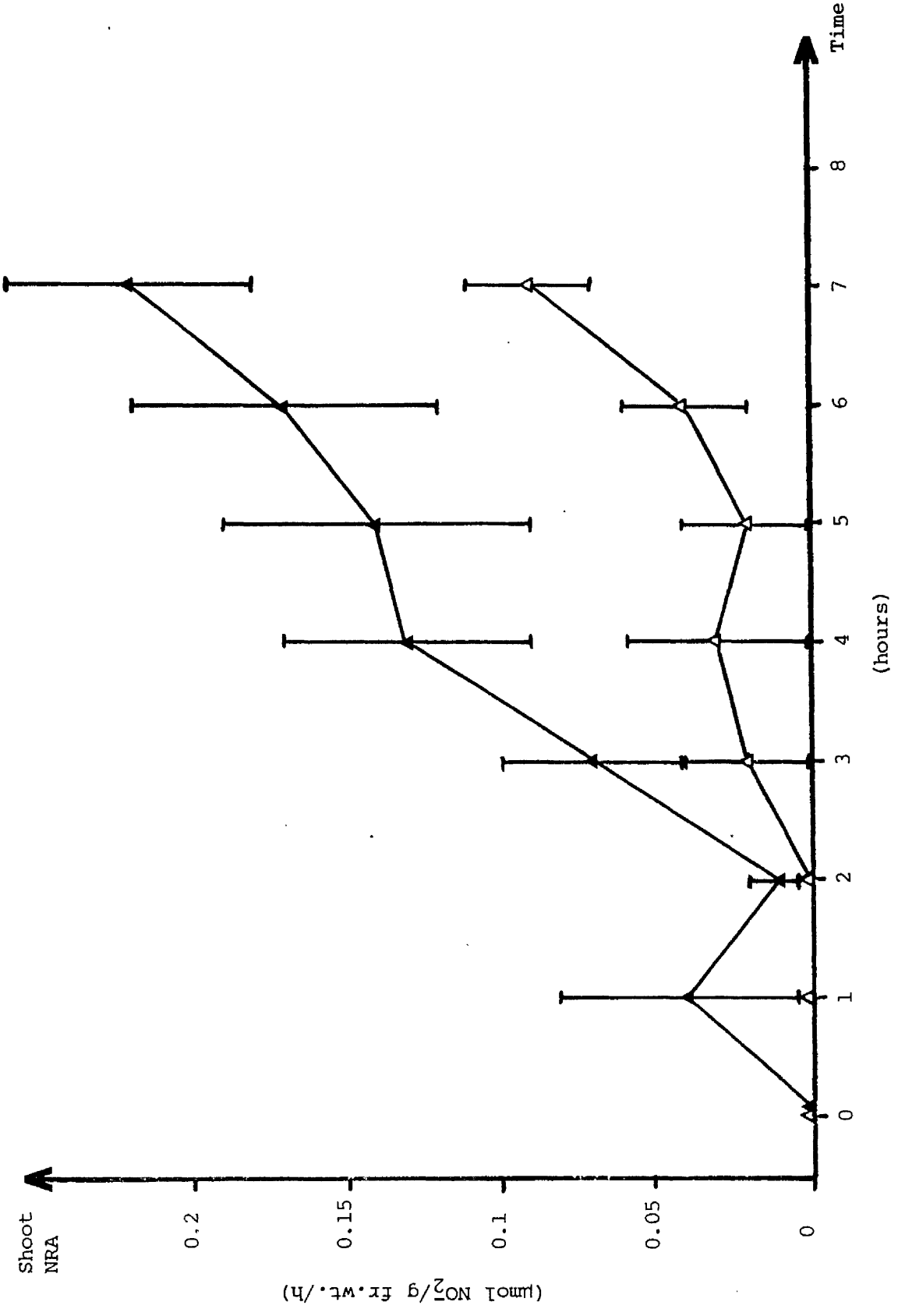
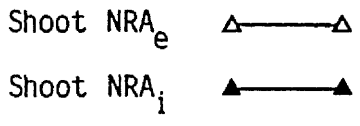

Shoot
NRA
0.2
0.15
0.1
0.05
0
(μmol NO2/g fr.wt./h)
0
1
2
3
4
5
6
7
8
Time
(hours)

FIGURE 23

Graph of shoot NRA_e and NRA_i versus time (days) after supplying NO_3^- to plants previously grown without NO_3^-.



Each point represents the mean of 5 replicates
Error bars mark standard deviations of means

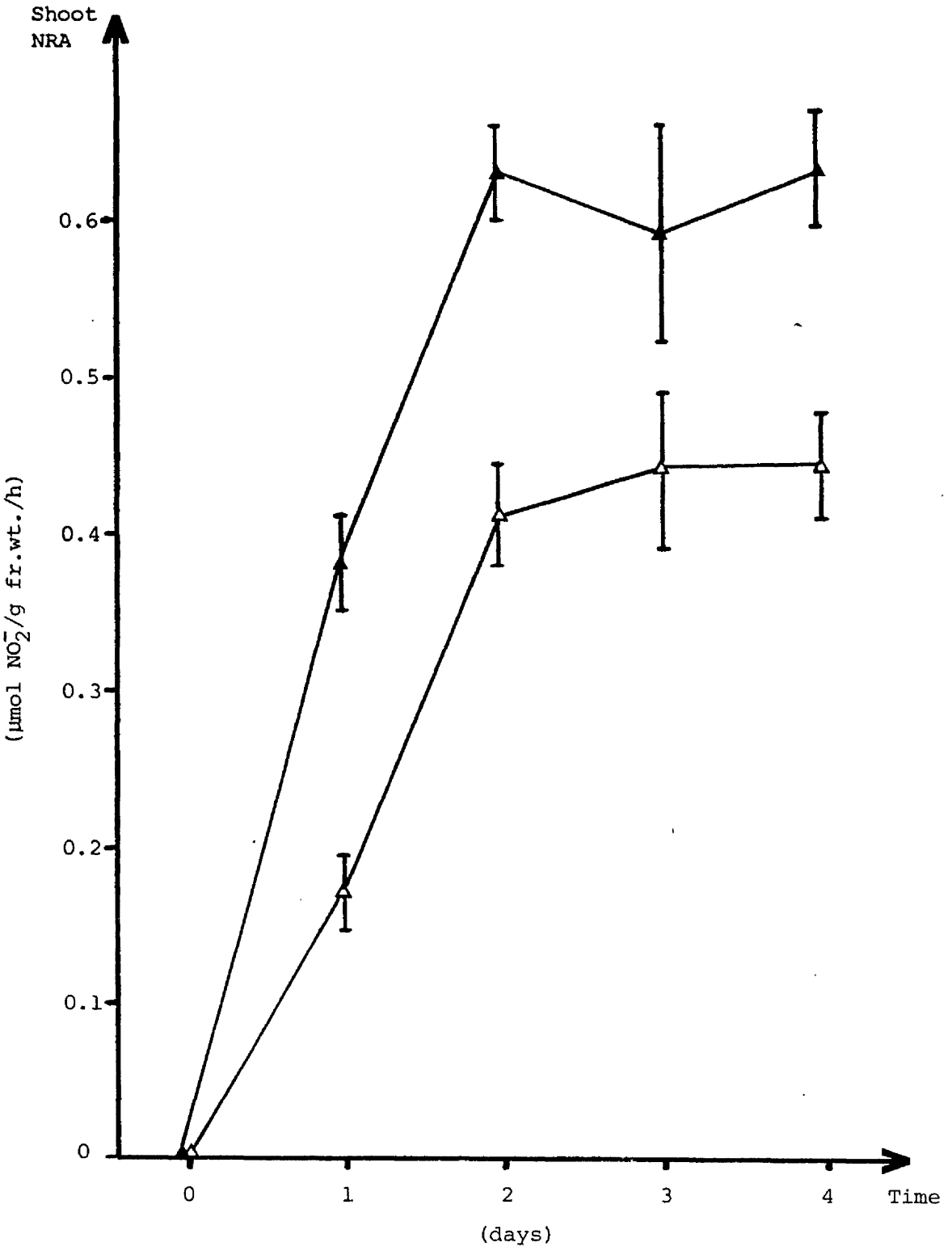
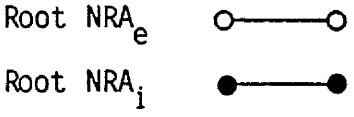
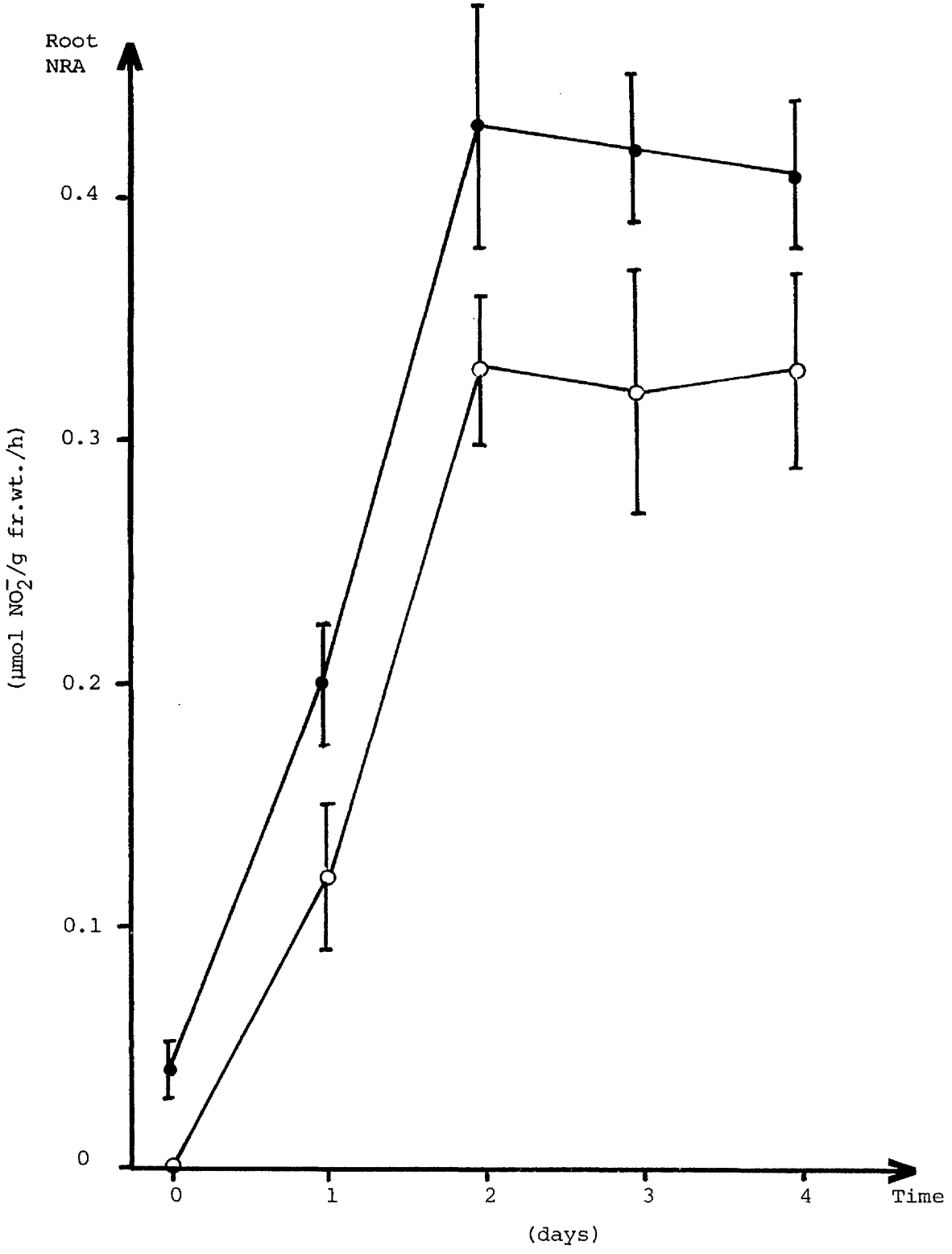

Shoot
NRA
0.6
0.5
0.4
0.3
0.2
0.1
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
3
4
Time
(days)

FIGURE 24

Graph of root NRA_e and NRA_i versus time (days) after supplying NO_3^- to plants previously grown without NO_3^-



Each point represents the mean of 5 replicates
Error bars mark standard deviations of means


Root
NRA
0.4
0.3
0.2
0.1
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
3
4
Time
(days)

synthesis of NR. With no new enzyme being produced, NRA declines as the enzyme originally present is degraded or inactivated. The rate of decline in NRA was therefore studied when barley plants were transferred from a NO_3^- - containing solution to one containing no NO_3^-.

Plants were grown as described earlier (section 4.2) until they reached the 3rd leaf stage, supplied with a NO_3^- containing nutrient solution. This solution was then flushed from the pots until water draining from the pots was free from NO_3^-. Plants were then supplied with NO_3^- - free nutrient solution. Shoot and root samples were assayed immediately for NRA_e and NRA_i, and at 24 hour intervals thereafter.

Twenty-four hours after the NO_3^- supply was withdrawn, shoot NRA_e and NRA_i were unchanged from their initial levels but thereafter decreased to reach low levels within 7 days after removal of NO_3^- nutrition (Figure 25). Shoot NRA_e and NRA_i did not start to decline immediately NO_3^- supply was stopped since substrate (NO_3^-) for NR may have been transferred from vacuolar storage pools to metabolically available cytoplasmic pools. NRA then declined when this NO_3^- supply was exhausted.

Hewitt (1982) found NRA to remain at initial levels for 2 days after cessation of NO_3^- nutrition but other workers have observed that leaf NRA declined by about 50% within 24 hours of removal of NO_3^- (Afridi & Hewitt, 1962 and Bowerman & Goodman, 1971). Melzer *et al.*, (1984) found that leaf NO_3^- content fell to very low levels in the 3 days after removal of NO_3^- nutrition from *Rumex* plants, during which time leaf NRA also declined markedly.

In contrast to shoot NRA, root NRA_e and NRA_i were only about 40% of their initial levels 24 hours after withdrawal of NO_3^- supply.

FIGURE 25

Graph of shoot NRA_e and NRA_i versus time after removal of NO_3^- nutrition

Shoot NRA_e △——△

Shoot NRA_i ▲——▲

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means

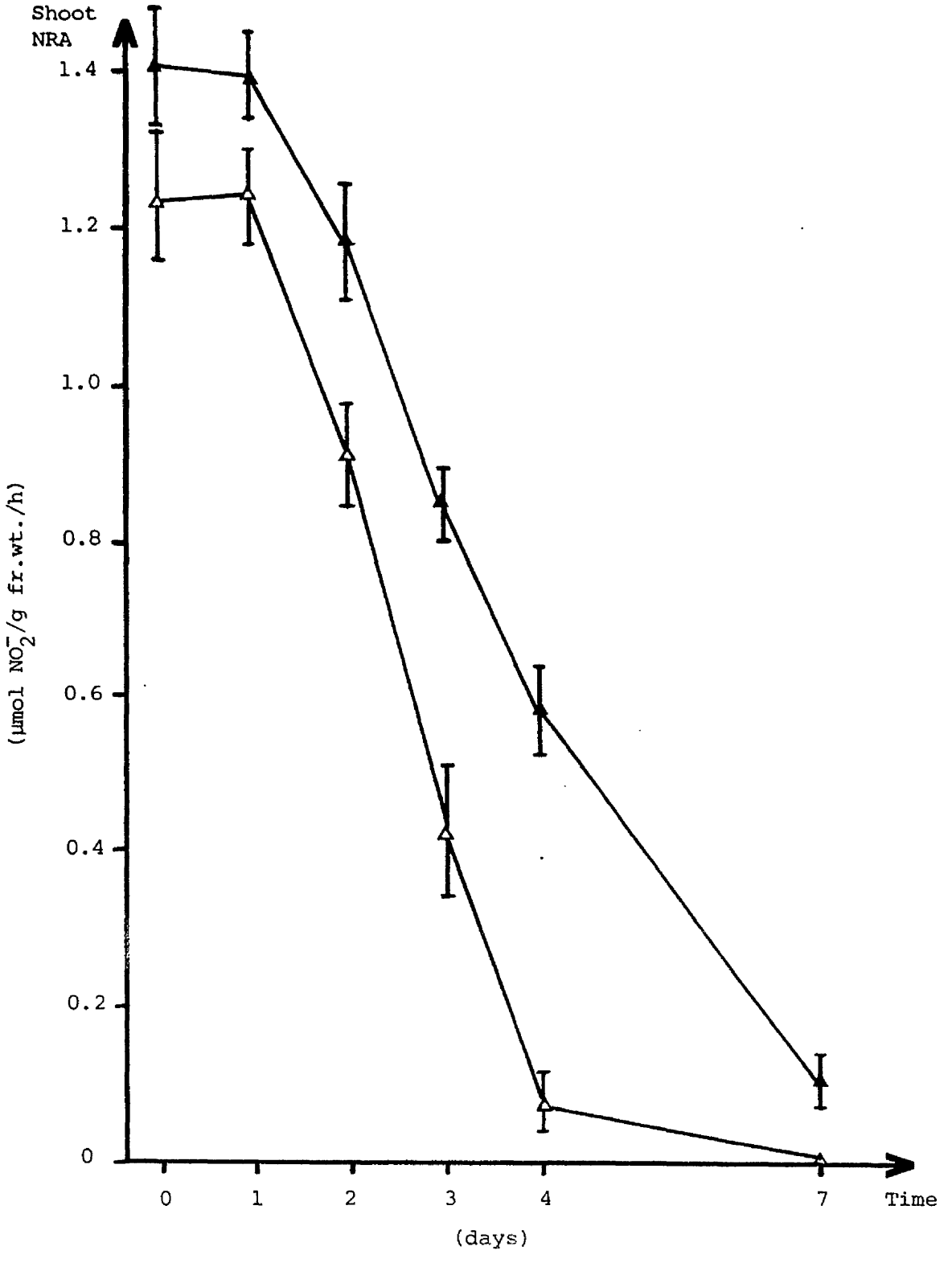
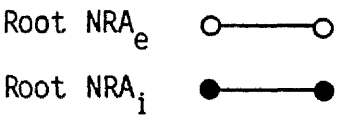

Shoot
NRA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
(µmol NO_2^-/g fr.wt./h)
0
1
2
3
4
7
Time
(days)

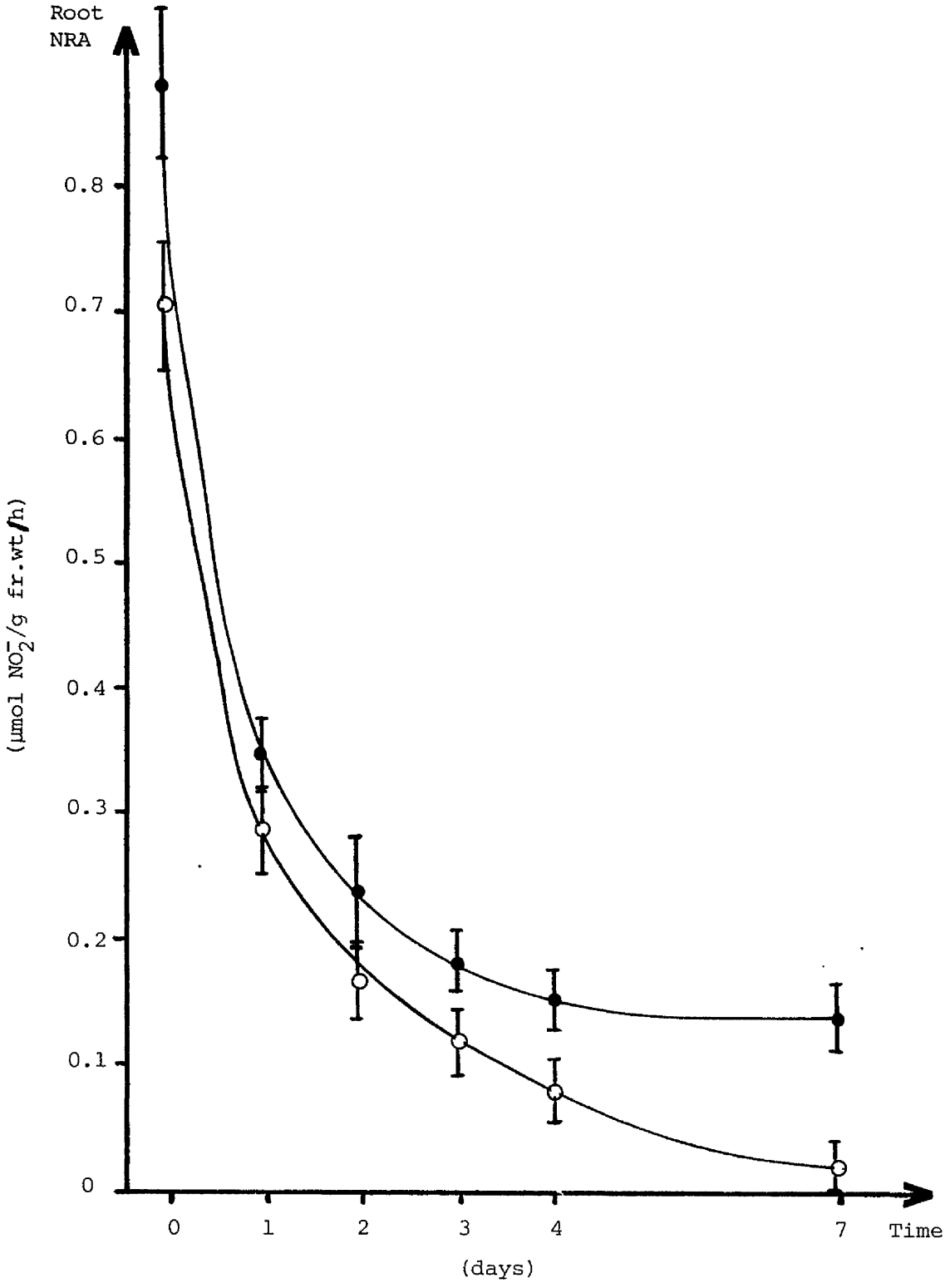
FIGURE 26

Root NRA_e and NRA_i versus time after removal of NO_3^- nutrition



Each point represents the mean of 6 replicates

Error bars mark standard deviations of means


Root
NRA
(µmol NO_2^-/g fr.wt/h)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
7
Time
(days)

Root NRA_e and NRA_i then declined to low levels within 7 days after removal of NO_3^- nutrition (Figure 26). It is possible that NO_3^- stored in roots was not available to NR or that root NO_3^- was transported *via* the xylem to maintain shoot NRA in preference to root NRA.

4.5.5 NH_4NO_3 nutrition

Nitrogen is absorbed preferentially as NH_4^+ from NH_4NO_3 solutions by many plant species (Pate, 1973; Frith & Nicholas, 1975 and Rao & Rains, 1976). When NH_4^+ is absorbed from a complete nutrient solution containing an all NH_4^+-N source, pH decreases as a result of OH^- import or H^+ export by the plant roots to maintain the cation/anion balance. In solutions containing an all NO_3^--N source, solution pH increases as a result of an excess of nutrient anions being taken up. In NH_4NO_3 solutions, if NH_4^+ is preferentially absorbed by plants, solution pH falls as time passes (Bigg & Daniel, 1978).

Much controversy exists over the effects of NH_4^+ on NRA. Some workers have proposed NH_4^+ to be an end-product repressor or inhibitor of NR (Smith & Thomson, 1971, Stewart, 1972 and Orebamjo & Stewart, 1975); while others have shown NH_4^+ to promote or stimulate NR induction (Schrader & Hageman, 1967; Bayley *et al.*, 1972 and Mohanty & Fletcher, 1976).

To study these effects then, barley plants were allowed to deplete the nitrogen from a complete nutrient solution originally containing equimolar amounts of NH_4^+ and NO_3^-. Rates of NH_4^+ and NO_3^- uptake were studied as was the drift in solution pH and variations in shoot NRA_e.

Plants were grown as described in section 4.2, but with a complete nutrient solution containing 10mg NH_4^+ - Nl^{-1} and 10mg NO_3^--Nl^{-1}.

Solutions were replaced every 2-3 days to prevent nutrient depletion and plants were grown until they reached the 3rd leaf stage, when they were then allowed to deplete the solutions of nitrogen over a further period of 3 weeks. Solutions were analyzed regularly for NH_4^+ and NO_3^-, pH was monitored and shoot samples were taken for NRA_e determination.

Figure 27 shows graphs of solution NH_4^+ and NO_3^- concentrations and shoot NRA_e over the depletion period. Under the conditions used here, plants utilised NH_4^+ in preference to NO_3^-, with virtually no NO_3^- being lost from the solution in the first 10 days of the depletion period.

Some oxidation of NH_4^+ to NO_3^- may have been caused by rhizosphere bacteria, and this might have maintained the high NO_3^- concentration despite possible NO_3^- absorption by plants. However, the relatively small biomass of bacteria and low rates of oxidation in nutrient solution probably made this a minor effect.

As the experiment progressed, solution pH fell from its original level (pH 6.3) to pH 5.8 by day 7, and to pH 5.3 by day 15. During the period day 15 to day 21, solution pH remained at 5.3, but by day 24 the trend had reversed and pH had risen to 5.4. The pH variation observed can be explained as the result of preferential NH_4^+ uptake and concomitant H^+ release from roots to solution (Breteler & Smit , 1974 and Mengel et al., 1983). The slight increase in pH observed at the end of the experiment is indicative of NO_3^- uptake with associated OH^- release into solution.

Shoot NRA_e was fairly constant during the first 14 days of the experiment, but then increased to a maximum by day 24. NH_4^+ appeared to exhibit a repressive or inhibitory effect on NO_3^- uptake or NRA_e

FIGURE 27

Nutrient solution NH_4^+ and NO_3^- concentrations and shoot NRA_e versus time after the start of the nutrient depletion period

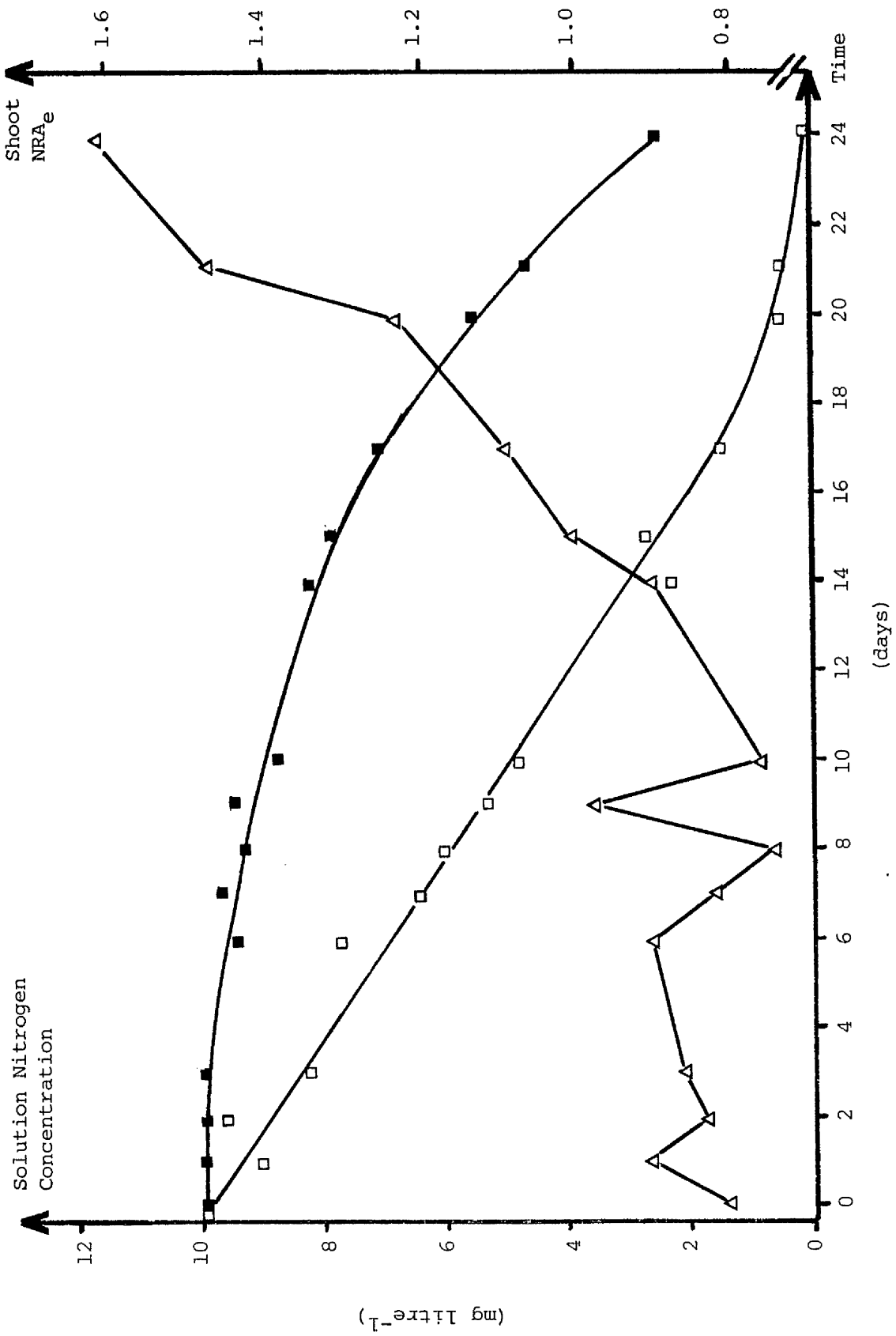
NO_3^- concentration ■——■

NH_4^+ concentration □——□

Shoot NRA_e △——△

Each point represents the mean of 3 replicate NRA_e determinations.

Error bars omitted for clarity


(µmol NO_2^-/g fr.wt./h)
Shoot NRA_e
1.6
1.4
1.2
1.0
0.8
Time
(days)
0
2
4
6
8
10
12
14
16
18
20
22
24
Solution Nitrogen Concentration
(mg litre^{-1})
12
10
8
6
4
2
0

of barley. During the first 14 days of the experiment, NO_3^- concentration only fell from 10 to 8.5mg NO_3^--N l^{-1}, while NH_4^+ concentration decreased from 10 to 2mg NH_4^+-N l^{-1}, and preferential NH_4^+ uptake kept NRA_e below 1.0 µmol NO_2^- g^{-1} fresh weight h^{-1}. After this period, NO_3^- concentration declined more rapidly, with shoot NRA_e continuing to increase to a maximum by the end of the experiment. There was then only 25% of the original NO_3^- left in solution, but virtually all the NH_4^+ had been removed, along with its inhibitory or repressive effect on NO_3^- uptake or NR.

4.5.6 Field Observations

NR activities have been used as potential indicators of nitrogen status of field crops (Bar-Akiva & Sternbaum, 1965, Shaked et al., 1974 and Oosterhuis and Bate, 1983). NAC ratios have also been used as indicators of the degree of nitrogen limitation in plants, where NAC = NRA_i/NRA_e (Shaked et al., 1974 and Davison & Stewart, 1983). Preliminary work using NAC ratios as indicators of nitrogen status of winter cereal crops has produced promising results (Verstraeten, 1983); although Sylvester-Bradley (1982) concluded that NRA was too labile to be of use as an indicator of crop nitrogen status.

Some preliminary field sampling was therefore carried out to assess the usefulness of the NAC ratio as an indicator of crop nitrogen status and to investigate the seasonal variation in NRA of field grown barley plants.

Shoot samples were taken at intervals over winter/spring 1983-84 from a commercially grown winter barley crop (variety Igri). Leaves of 20 plants were taken from 8 random sites during the middle of the photoperiod on dry, sunny days. Samples were then assayed for

NRA_e and NRA_i, and NAC ratios were calculated.

Soil samples were also taken (to a depth of 34cm from the surface) and extracted with 2M KCl (20g of moist soil were shaken with 100ml of 2M KCl for 1 hour at room temperature). The extract was then filtered and the filtrate was analysed for NO_3^- (phenoldisulphonic acid method, Appendix 9) and NH_4^+ (Nessler's method, Appendix 8). Soil moisture contents were also determined and soil NO_3^- and NH_4^+ concentrations were then expressed as $\mu g\ N\ g^{-1}$ O.D. soil.

TABLE 15: Shoot NRA_e, NRA_i and NAC values of a field crop of barley over the winter/spring period

Sampling date	Shoot NRA_e ($\mu mol\ NO_2^-\ g^{-1}$ fresh weight h^{-1})	Shoot NRA_i	NAC (ratio)
Dec. 12 1983	1.0_a	1.6_c	1.7_g
Jan. 19 1984	0.8_a	1.5_c	2.1_{gh}
Feb. 23 1984	1.0_a	2.1_d	2.3_{gh}
Mar. 16 1984	1.1_a	2.7_e	2.6_h
N fertiliser applied on Mar. 19 and Apr. 2			
Apr. 13 1984	3.2_b	3.9_f	1.2_i
May 5 1984	2.8_b	3.6_f	1.3_i

Results are means of 8 replicates.
Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

N fertiliser (NH_4NO_3) was applied as a top-dressing at rates of 80kg N ha^{-1} on March 19 and 60kg N ha^{-1} on April 2.

Shoot NAC ratios increased from Dec. 12 to Mar. 16 (Table 15), during which time, soil NO_3^- concentrations decreased to very low levels, and soil NH_4^+ concentrations increased only slightly (Table 16).

TABLE 16: Soil NO_3^- and NH_4^+ levels under a crop of winter barley over the winter/spring period

Sampling date	Soil NO_3^- - N	Soil NH_4^+ - N
	($\mu g\ N\ g^{-1}$ O.D. soil)	
Dec. 12	3.8_a	0.5_a
Jan. 19	2.7_a	0.6_{ab}
Feb. 23	1.5_b	0.8_{ab}
Mar. 16	0.4_c	0.9_b
Supplemental N fertiliser applied on Mar. 19 and Apr. 2		
Apr. 13	1.4_b	2.9_c
May 5	1.4_b	1.6_d

Results are means of 8 replicates.

Results followed by the same letter(s) (compared vertically) are not significantly different at the 5% level.

Increases in shoot NAC ratios indicated an increasing crop demand for nitrogen i.e. the higher the NAC ratio, the lower the crop N status. High NAC ratios indicated that the crop would respond favourably to nitrogen fertiliser.

After top-dressing with nitrogen fertiliser, NRA increased markedly, demonstrating substrate induction of NR on a field scale, an effect also seen by Croy & Hageman (1970) and Hatam (1978). After nitrogen top dressing, the NAC ratio was low (~ 1.2), as were soil NO_3^- and NH_4^+ concentrations (perhaps due to rapid crop uptake). The marked decrease in NAC ratio after nitrogen fertiliser application indicated that the nitrogen demand of the crop had, to a large extent, been satisfied.

4.5.7 Conclusions

The following conclusions were reached in the study of effects of nitrogen nutrition on barley NRA:

a) Plants grown without NO_3^- showed no NRA_e, although very low levels of NRA_i could be induced by NO_3^- during a 1 hour assay of excised plant material (section 4.5.2).

b) Low levels of shoot NRA_e were detected within 3 hours after feeding NO_3^- to plants previously grown without NO_3^-, while shoot NRA_i was detected within 1 hour. Shoot and root NRA_e and NRA_i increased to reach maximum, plateau levels 48 hours after supplying NO_3^- to plants previously grown without NO_3^- (section 4.5.3).

c) Shoot NRA_e and NRA_i remained at initial levels for 24 hours after cutting off NO_3^- nutrition before declining to very low levels within 7 days. By contrast, root NRA_e and NRA_i declined by about 60% within 24 hours, and then fell to very low levels within 7 days after stopping the NO_3^- supply (section 4.5.4).

d) When plants were allowed to deplete an NH_4NO_3 solution, NH_4^+ was utilised in preference to NO_3^-. Shoot NRA_e remained constant until about 80% of the NH_4^+ was utilised and then increased markedly as NO_3^- uptake increased when repressive effects of NH_4^+ on NO_3^- uptake or NR were removed (section 4.5.5).

e) In a preliminary field study of a winter barley crop, shoot NAC ratios increased during the period December to March, indicating an increasing crop demand for nitrogen. After top-dressing with NH_4NO_3 fertiliser, shoot NAC ratio decreased to close to 1, indicating that crop nitrogen demand had, to a large extent, been satisfied (section 4.5.6).

4.6 Controlled environmental conditions

4.6.1 Introduction

The effect of the following factors, under controlled environmental conditions, on the growth, composition and NRA of barley plants were investigated:

a) NO_3^- concentration (section 4.6.3)

b) Nitrogen form (section 4.6.4)

c) Nutrient solution pH (section 4.6.5)

d) Root temperature (section 4.6.6)

e) Shoot temperature (section 4.6.7).

4.6.2 Materials and Methods

A large (5 x 3m) coldstore was converted into a controlled environment room. Benches, fluorescent lights and 3 large nutrient solution reservoir tanks (150 l each) were installed. Each tank was fitted with a pump to lift solutions up main pipes, along benches and through small feeder tubes to supply six 40 x 25 x 7.5cm trays. The trays were fitted with overflows which drained solution back to the tanks, hence forming a continuously recirculating system. The solution depth in each tray was 6cm and solutions flowed at a rate of about 0.7 l min^{-1} through each tray. The eighteen trays (6 replicates of each of 3 treatments) were then arranged in a randomised block design on the bench. Each tank was fitted with thermostats and heating/refrigeration coils to maintain the desired temperature. Air temperature was also thermostatically controlled.

Barley seeds (variety Igri) were soaked in water (5 hours), germinated on moist paper (40 hours in the dark at room temperature)

and 20 chitted seeds were then planted in each of 72 pots (13cm diameter) of perlite. Four pots were then stood on supports in each tray so that the solution depth in the pots was 3cm. Each tray then constituted 1 replicate.

Plants were grown for 5 days at a temperature of $30^{o}C$ under lights of intensity 7klx at the pot surfaces, with 12 hour photoperiods and a nitrogen free nutrient solution. The solution had a pH of 6.5, a conductivity of 1500 $\mu S\,cm^{-1}$ and contained (mg l^{-1}):

P	40	Mg	40	B	0.8	Zn	0.4
K	320	Fe	4	Mo	0.08		
Ca	160	Mn	2	Cu	0.4		

After the 5 day period, seedlings were thinned to leave 15 plants per pot (60 plants per replicate) and the temperature was reduced to $10^{o}C$ over the next 5 days. Three chosen treatments were then imposed, a nitrogen supply was added and solution conductivities were adjusted to 2000 $\mu S\,cm^{-1}$ with $CaSO_4$ (if necessary). Plants were then grown under the treatments until they reached the 4th leaf stage (about 21 days).

During this time, recirculating solutions were analysed every 2-3 days for NO_3^- (phenoldisulphonic acid method, Appendix 9) and NH_4^+, if appropriate (Nessler's method, Appendix 8). Solution conductivity and pH were also monitored. Initial nitrogen concentrations, pH's and conductivities were maintained throughout each experiment by appropriate adjustment after analysis.

All the shoots and roots were harvested when plants reached the 4th leaf stage. Most of the roots had grown out of the pots into the flowing solution and were easily harvested. The rest of the roots were recovered from the perlite and washed free of adhering

particles. Roots were then centrifuged in a basket (1025 g for 3 minutes) to remove surface water. Next, shoot fresh weights were recorded, shoot and root samples were assayed for NRA_e and NRA_i and NAC ratios were calculated.

The remainder of each sample was dried and dry weight and % dry matter were calculated. Dried samples were then finely milled and used for subsequent analysis for reduced nitrogen (Appendix 7), and for preparation of cold-water extracts (0.2g dry material shaken with 100ml cold water for 2 hours). After filtering the extracts, filtrates were analysed for NO_3^- (Appendix 9), water soluble carbohydrates (Appendix 10), and L-malate (Appendix 11). Finally, total nitrogen was expressed as the sum of reduced nitrogen and tissue NO_3^- nitrogen.

4.6.3 NO_3^- concentration

Introduction

Since NR is a substrate inducible enzyme, it is likely that the NO_3^- concentration supplied to plants will affect NRA. Accordingly, the effect of the NO_3^- concentration supplied (in the range 0 to 64mg NO_3^- - $N\,l^{-1}$) on the growth, composition and NRA of barley plants was investigated.

Experimental details

Plants were raised in the controlled environment room as described previously (section 4.6.2) and a series of seven NO_3^- concentrations covering the range 0-64mg NO_3^- - $N\,l^{-1}$ were supplied and maintained in a series of 3 experiments (since only 3 treatments could be investigated at any one time). Common treatments were used between

experiments to provide comparisons over the whole range of concentrations studied. The nitrogen concentrations supplied and maintained were:

Experiment 1: 0, 2 ± 0.5 and 4 ± 0.8mg NO_3^- - Nl^{-1}

Experiment 2: 4 ± 0.8, 8 ± 1 and 16 ± 1mg NO_3^- - Nl^{-1}

Experiment 3: 16 ± 1, 32 ± 2 and 64 ± 4mg NO_3^- - Nl^{-1}

All plants were grown under the same conditions of solution pH (pH 6.8 - 6.3), solution conductivity (1800-2200 $\mu S\,cm^{-1}$) and temperature (10 ± 1°C) for 21 days, and were then harvested and analysed as described earlier (section 4.6.2).

Results

By the end of the treatment period, the plants had reached the 4th leaf stage. Plants grown with 4 to 64mg NO_3^- - Nl^{-1} showed no visible signs of nutrient deficiencies. Leaves of plants grown with 2mg NO_3^- - Nl^{-1} were, however, less green than those grown with higher NO_3^- concentrations and had some necrotic spots on lower leaves. Plants grown without NO_3^- were very chlorotic and had many necrotic spots on older leaves.

Nutrient solution pH tended to increase during the experiments, as a result of excess nutrient anion absorption, and H_2SO_4 was added to keep pH within the range 6.8-6.3.

The amounts of NO_3^- which had to be added to maintain initial concentrations are listed in Table 17.

Inevitably, small leaks developed in the recirculating system, but the amount of solution lost could not be quantified, since evaporation of water also took place and reservoirs were topped-up with water prior to analysis. Leakage of solution meant that solutions

TABLE 17: Amounts of NO_3^- needed to maintain initial NO_3^- concentrations

Experiment	Treatment (mg NO_3^- - N l^{-1})	NO_3^- added (mg NO_3^- - N)
1	0	0
1	2	180
1	4	310
2	4	300
2	8	400
2	16	550
3	16	590
3	32	620
3	64	630

at analysis were less concentrated in NO_3^- than was due to depletion by the plants. The amount of solution lost was small, probably less than 5%, but different amounts lost from each treatment meant that the amounts of nitrogen which had to be added to maintain initial concentrations served only as estimates of amounts of nitrogen actually utilized by the plants.

In addition to errors caused by loss of solution, there are potentially large errors in the amounts of NO_3^- added, since they were based on 8 separate analyses.

Greater amounts of NO_3^- had to be added as the NO_3^- concentration being maintained increased (Table 17). Amounts of solution lost from, and errors incurred in the analysis of, each treatment were probably similar, and it is therefore likely that a greater amount of NO_3^- was actually utilised by plants as the NO_3^- concentration available increased.

Fresh and dry weights and % dry matter

Shoot fresh weight increased from a minimum, when no NO_3^- was supplied, to a maximum when > 16mg NO_3^- - N l^{-1} were supplied (Figure 28). The similarity of results from common treatments between experiments made it reasonable to follow trends over the whole range of NO_3^- concentrations tested.

Statistically significant increases in shoot dry weight (Figure 29) as related to NO_3^- concentration supplied were not found within individual experiments. Large increases in dry matter production of plants supplied with NO_3^- compared with those not fed NO_3^- were expected since, even when only 2mg NO_3^- - N l^{-1} were supplied, NO_3^- should have been available at all times and should not have limited growth. The possibility of NO_3^- depletion within the rooting medium was therefore investigated (Appendix 13).

Partial NO_3^- depletion was found, especially near the tops of pots, but it is likely that all plants supplied with nitrogen, even those in the 2mg l^{-1} NO_3^- - N treatment, always had some NO_3^- available.

Large standard deviations of means of shoot dry weights were observed. Such large deviations were surprising in plants grown under controlled environmental conditions, and so the variation between individual plants was investigated (Appendix 14). The considerable variation found within a population made it feasible for there to be large variations between populations and may account for large standard deviations of means found in some cases.

Although statistically significant increases in shoot dry weight were not found within individual experiments, the trend over all 3 experiments was of increasing shoot dry matter production as the NO_3^- concentration supplied was increased.

FIGURE 28

Shoot fresh weight versus NO_3^- concentration maintained in solution

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means
Composite data from 3 experiments

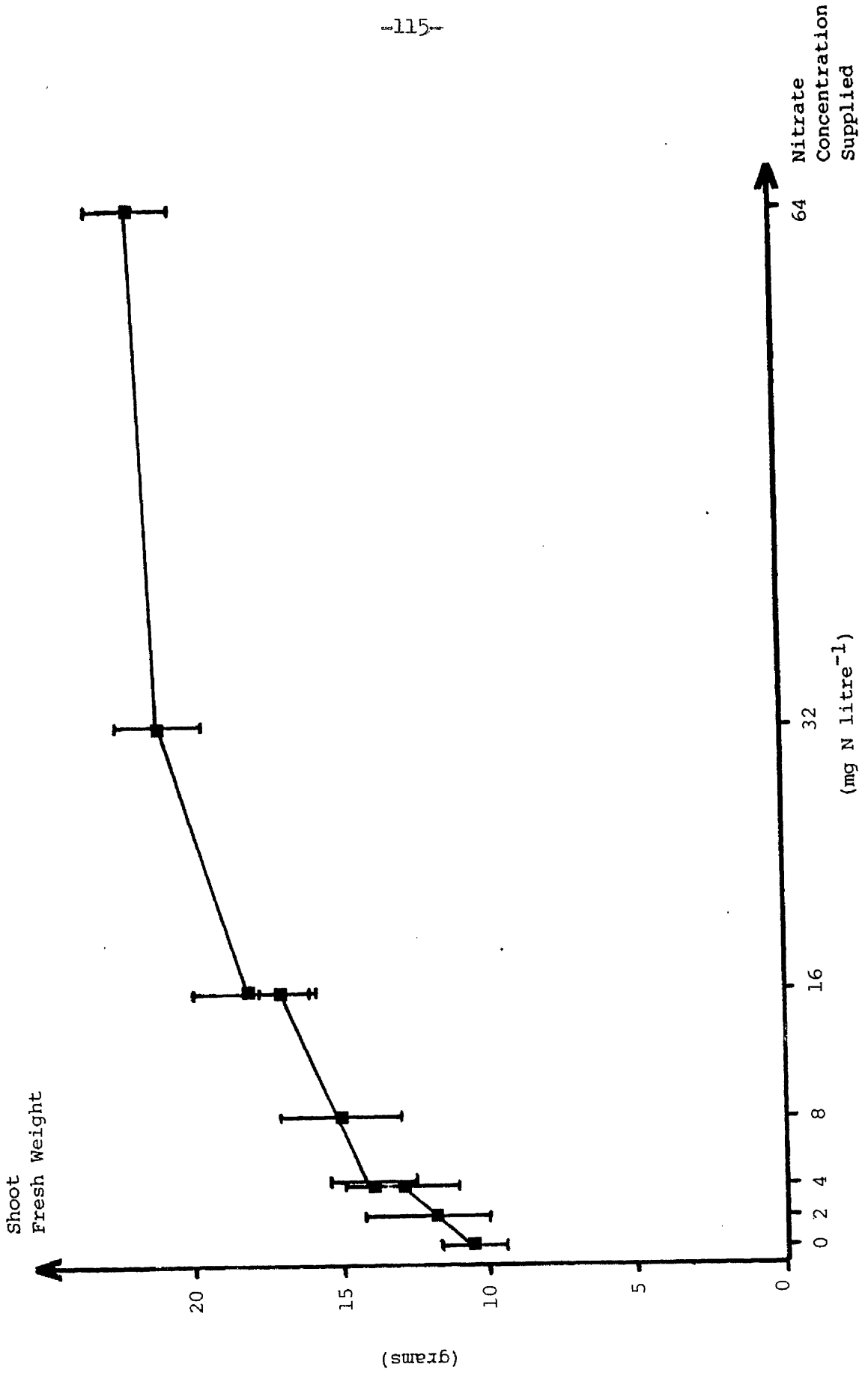

Shoot
Fresh Weight
(grams)
20
15
10
5
0
0 2 4 8 16 32 64
(mg N litre^{-1})
Nitrate
Concentration
Supplied

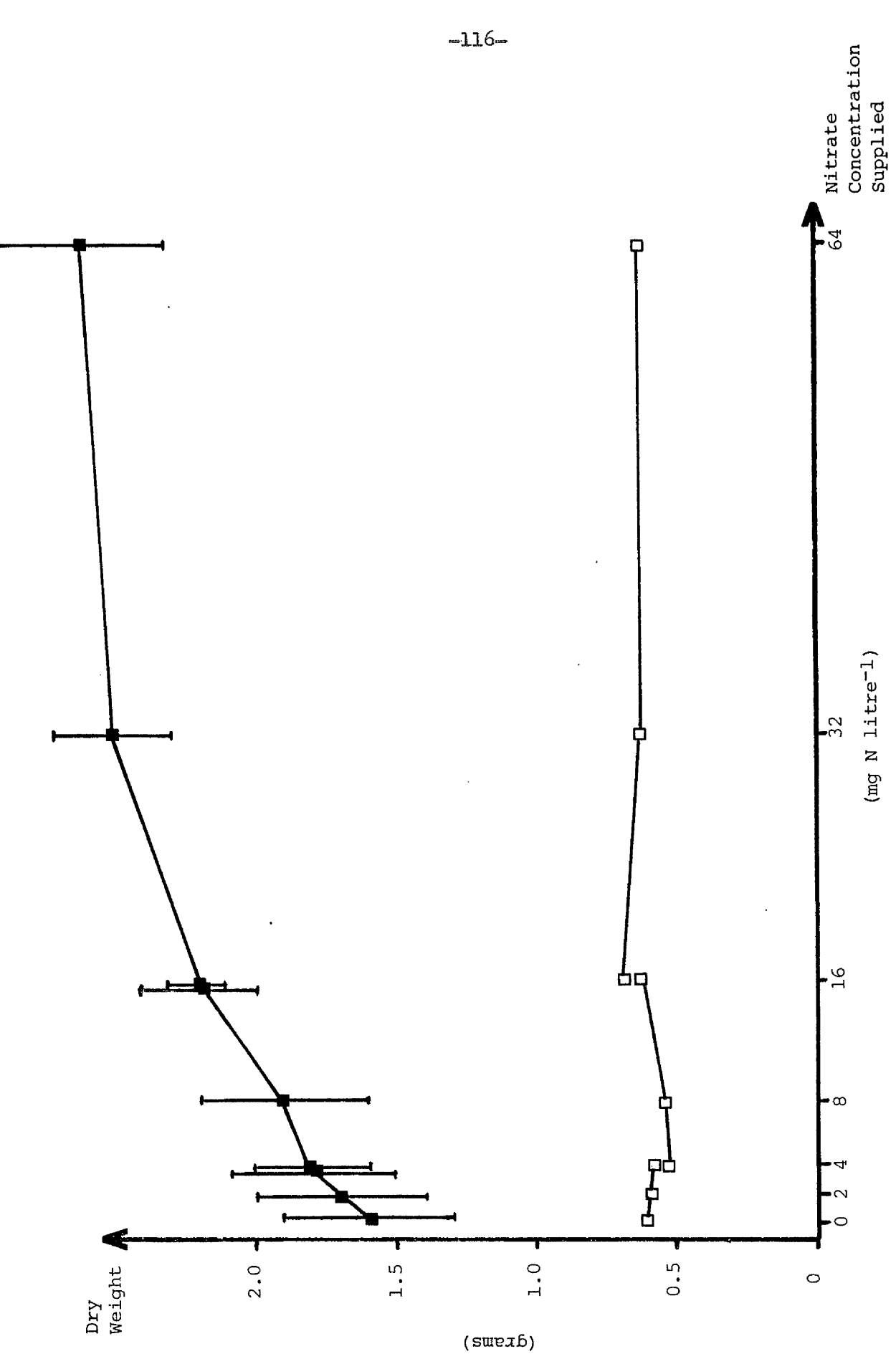
FIGURE 29

Shoot and root dry weight versus NO_3^- concentration maintained in solution

Shoot dry weight ■——■

Root dry weight □——□

Each point represents the mean of 6 replicates
Error bars mark standard deviation of means of shoot results
Composite data from 3 experiments


Dry Weight
(grams)
2.0
1.5
1.0
0.5
0
0 2 4 8 16 32 64
(mg N litre-1)
Nitrate Concentration Supplied

Increases in root dry matter recovered were not observed as the NO_3^- concentration supplied increased (Figure 29).

During harvesting fine roots were inevitably left in the perlite or lost during washing. Weight of roots recovered is therefore recorded and not total weight produced.

Differences in shoot % dry matter within individual experiments were not statistically significant (Figure 30), but the trend over all 3 experiments was of a decrease as NO_3^- concentration supplied increased.

NRA_e, NRA_i and NAC ratios

Shoot NRA_e and NRA_i increased asymptotically from zero, when no NO_3^- was supplied, to maximum, plateau levels when $\geqslant$ 16mg NO_3^--N l^{-1} were supplied (Figure 31). Other workers have found NO_3^- concentrations of 7 to 1400mg N l^{-1} to be required for maximal induction of NRA, depending on plant species and growing conditions (Bowerman & Goodman, 1971; Wallace, 1973 and Brunetti & Hageman, 1976). Trends in root NRA were similar to those in shoot NRA, although maximum root NRA levels were only about 50% of maximum shoot NRA (Figure 32).

NAC ratios could not be calculated for the no NO_3^- treatment, since NRA_e values were zero. Shoot NAC ratio decreased asymptotically from a maximum of 2.6 when 2mg NO_3^- - N l^{-1} were supplied, to a minimum value of 1 when $\geqslant$ 16mg NO_3^- - N l^{-1} were supplied (Figure 33). Differences in root NAC ratio were not statistically significant within individual experiments, but the overall trend (Figure 34) was similar to that of shoot NAC ratio.

Dry matter analysis

Shoot and root total N contents increased from a minimum, when no NO_3^- was supplied, to maximum, plateau levels when 64mg NO_3^- - N l^{-1}

FIGURE 30

Shoot % dry matter versus NO_3^- concentration maintained in solution

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means
Composite data from 3 experiments

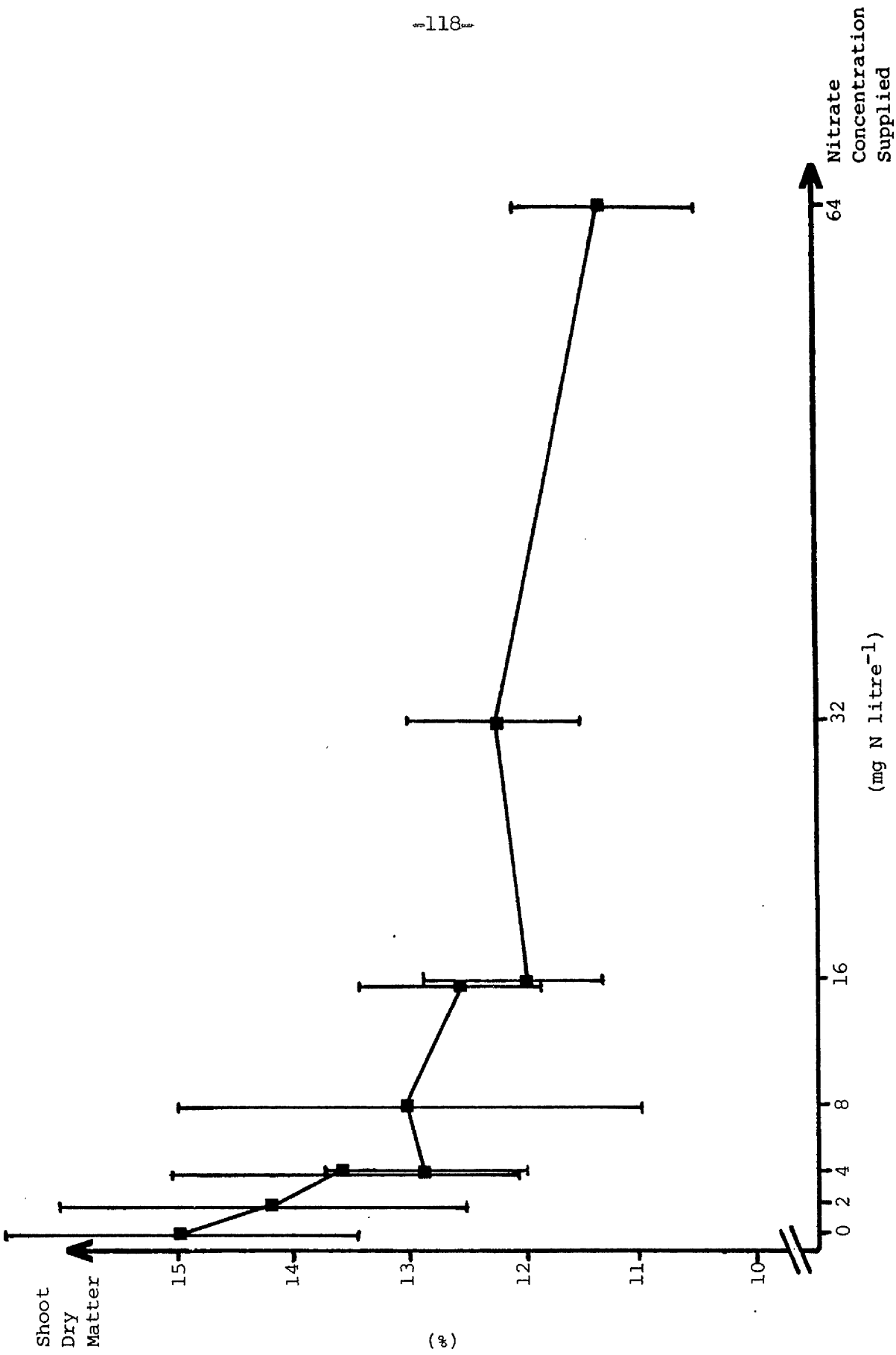
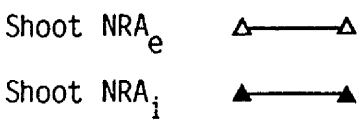

Shoot Dry Matter
(%)
15
14
13
12
11
10
0
2
4
8
16
32
64
(mg N litre^{-1})
Nitrate Concentration Supplied

FIGURE 31

Shoot NRA_e and NRA_i versus NO_3^- concentration maintained in solution



Each point represents the mean of 6 replicates
Error bars omitted for clarity but coefficients of variation were all < 12 %
Composite data from 3 experiments

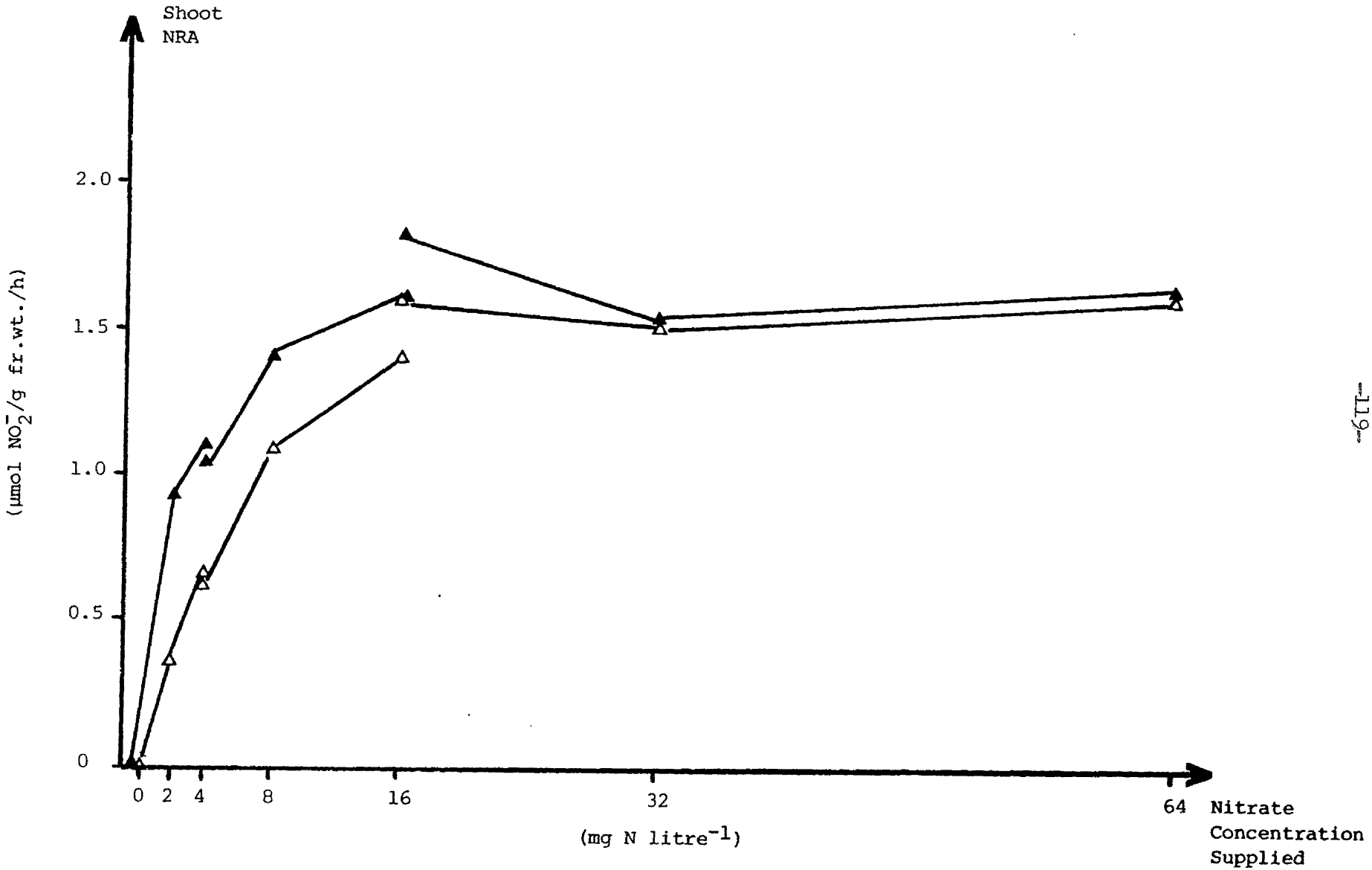
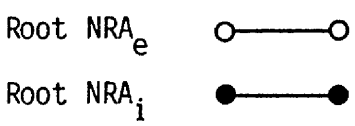

Shoot
NRA
2.0
1.5
1.0
0.5
0
(µmol NO_2^-/g fr.wt./h)
0
2
4
8
16
32
64
(mg N litre^{-1})
Nitrate
Concentration
Supplied

FIGURE 32

Root NRA_e and NRA_i versus NO_3^- concentration maintained in solution



Each point represents the mean of 6 replicates
Error bars omitted for clarity but coefficients of variation were all < 19%
Composite data from 3 experiments

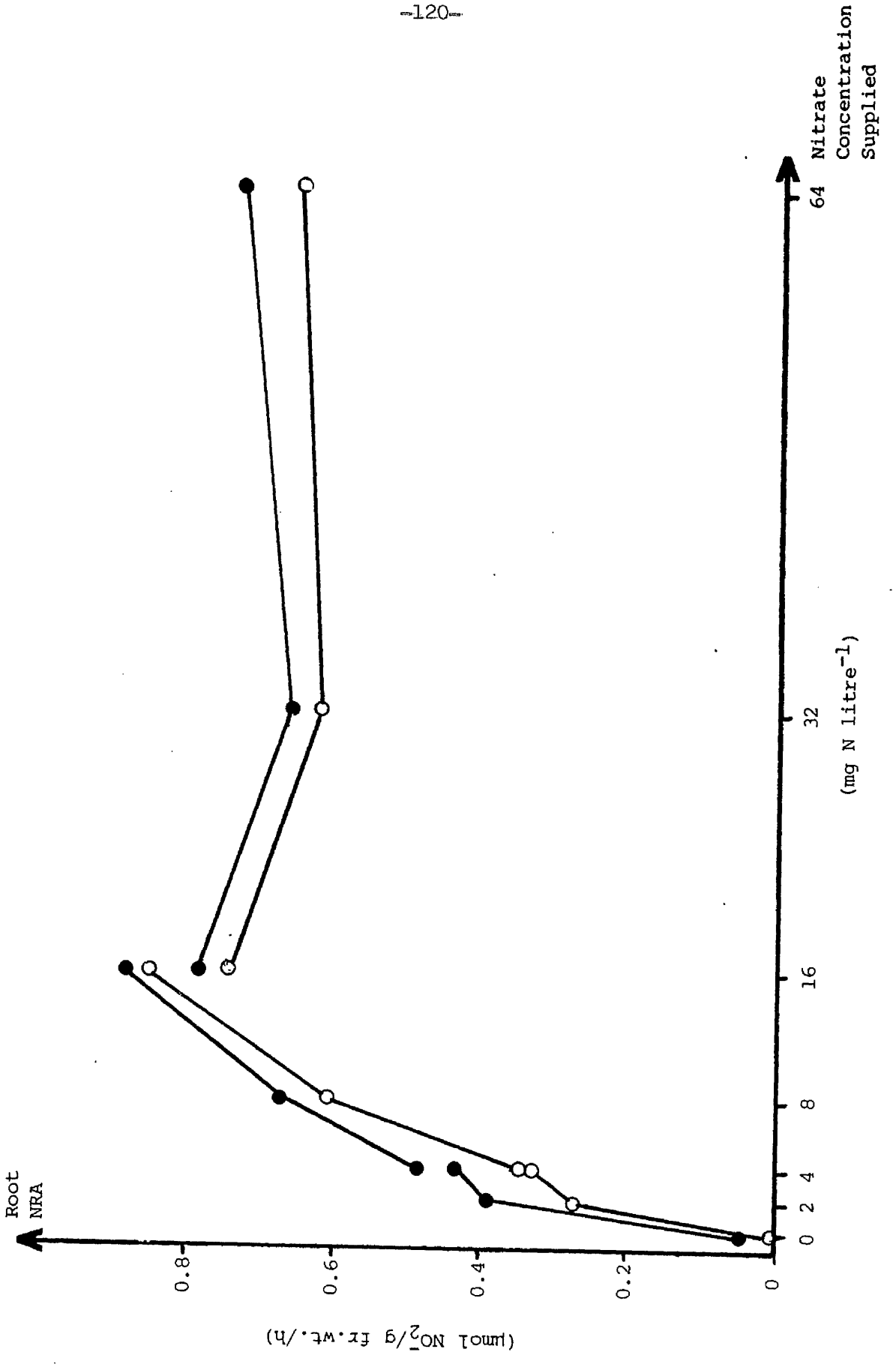

Root NRA
(μmol NO_2^-/g fr.wt./h)
0
0.2
0.4
0.6
0.8
0
2
4
8
16
32
64
(mg N litre^{-1})
Nitrate Concentration Supplied

FIGURE 33

Shoot NAC ratio versus NO_3^- concentration maintained in solution

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means
Composite data from 3 experiments

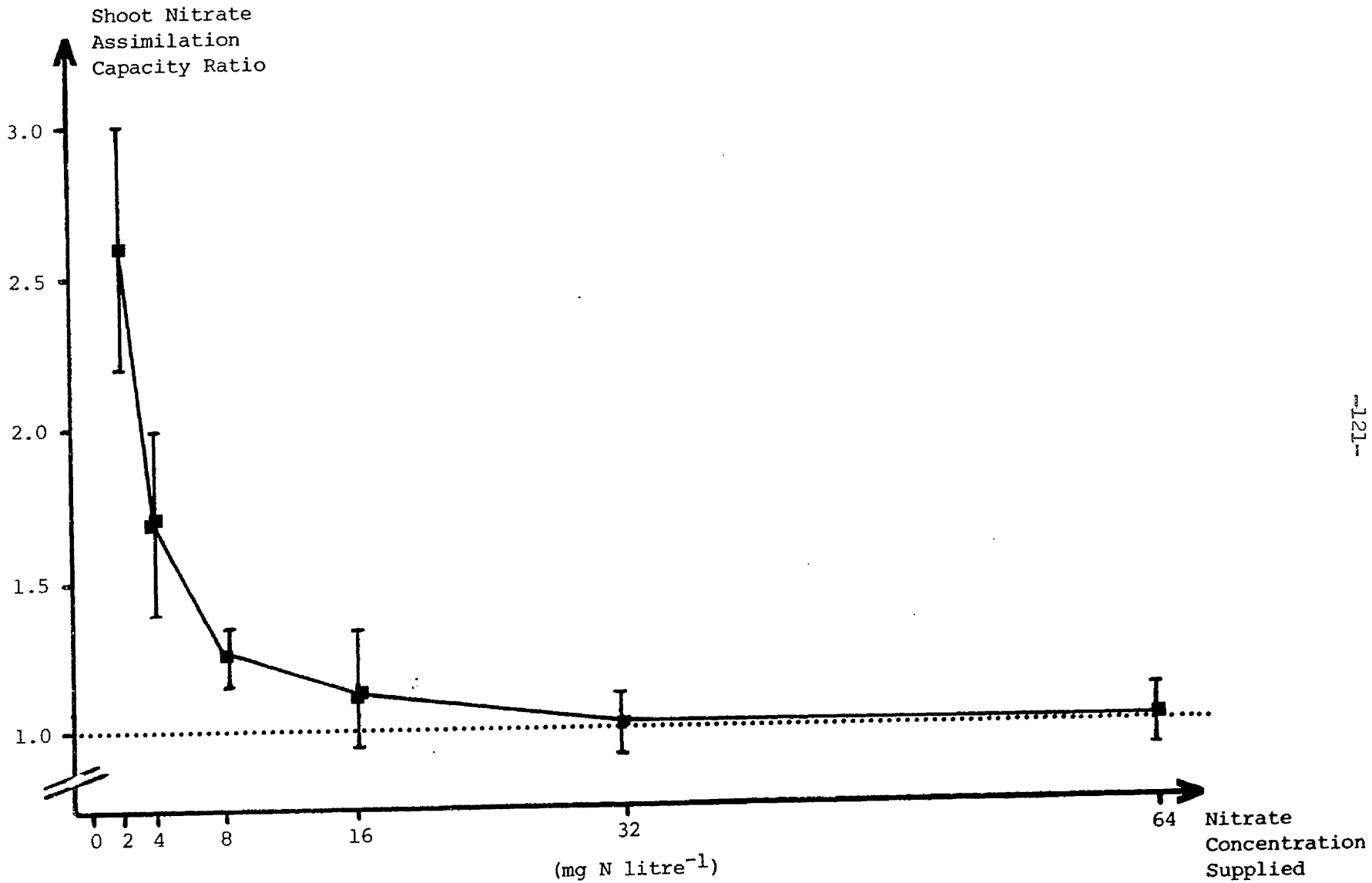
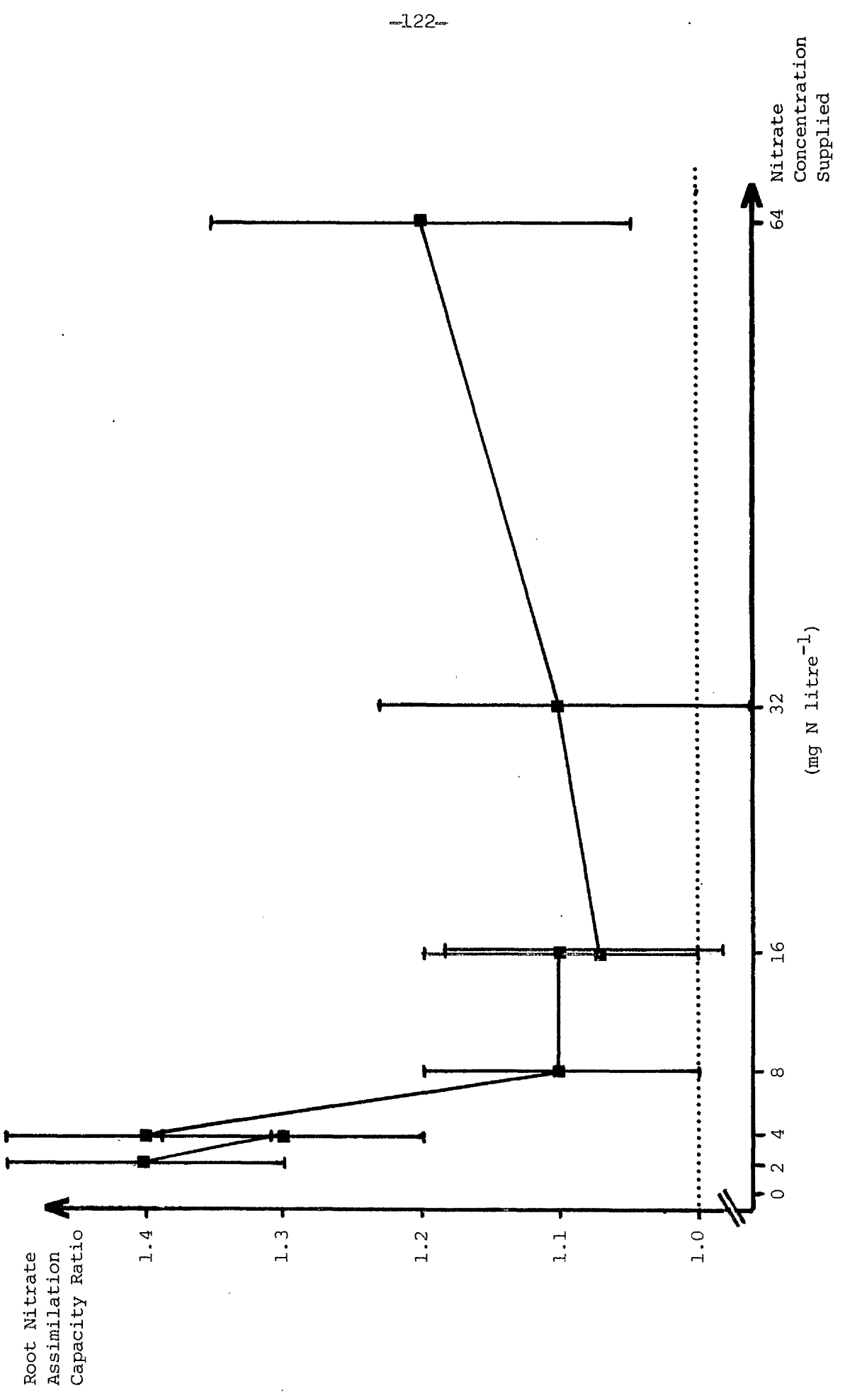

Shoot Nitrate
Assimilation
Capacity Ratio
3.0
2.5
2.0
1.5
1.0
0
2
4
8
16
32
64
(mg N litre^{-1})
Nitrate
Concentration
Supplied

FIGURE 34

Root NAC ratio versus NO_3^- concentration maintained in solution

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means
Composite data from 3 experiments


Root Nitrate Assimilation Capacity Ratio
1.4
1.3
1.2
1.1
1.0
0
2
4
8
16
32
64
Nitrate Concentration Supplied
(mg N litre^{-1})

were supplied (Figure 35).

Shoot and root tissue NO_3^- contents were of similar magnitude and increased from zero, when no NO_3^- was supplied, to a maximum at the highest solution NO_3^- concentration tested (Figure 36).

Shoot and root water soluble carbohydrate concentrations decreased asymptotically, from a maximum in plants not supplied with NO_3^-, to a minimum in plants fed $\geqslant$ 16mg NO_3^- - N l^{-1} (Figure 37). Decreased sugar contents were also observed as NO_3^- concentrations supplied to plants were increased by Radin et al., (1978).

Shoot and root L-malate concentrations increased, from a minimum in plants fed no NO_3^-, to a maximum in plants fed $\geqslant$ 16mg NO_3^- - N l^{-1} (Figure 38).

Conclusions

The following conclusions were reached in the study of the effects of NO_3^- concentration on growth, composition and NRA of barley plants:

a) More NO_3^- was taken up by plants as the NO_3^- concentration maintained at the root was increased.

b) Shoot fresh and dry weights increased as NO_3^- concentration supplied increased, while shoot % dry matter tended to decrease.

c) Shoot and root NRA_e and NRA_i increased from close to zero, when no NO_3^- was supplied, to maximum, plateau levels when $\geqslant$ 16mg l^{-1} NO_3^- - N was supplied. NAC ratios decreased from a maximum, when 2mg NO_3^- - N l^{-1} was supplied, to a minimum of about 1 when $\geqslant$ 16mg NO_3^- - N l^{-1} was supplied.

d) Shoot and root total N, NO_3^- - N and L-malate concentrations increased as the NO_3^- concentration supplied increased, while water soluble carbohydrate contents decreased.

FIGURE 35

Shoot and root total nitrogen content versus NO_3^- concentration maintained in solution

Shoot total N ■——■

Root total N □——□

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means of shoot results
Error bars omitted from root results for clarity, but coefficients of variation were all < 11%
Composite data from 3 experiments

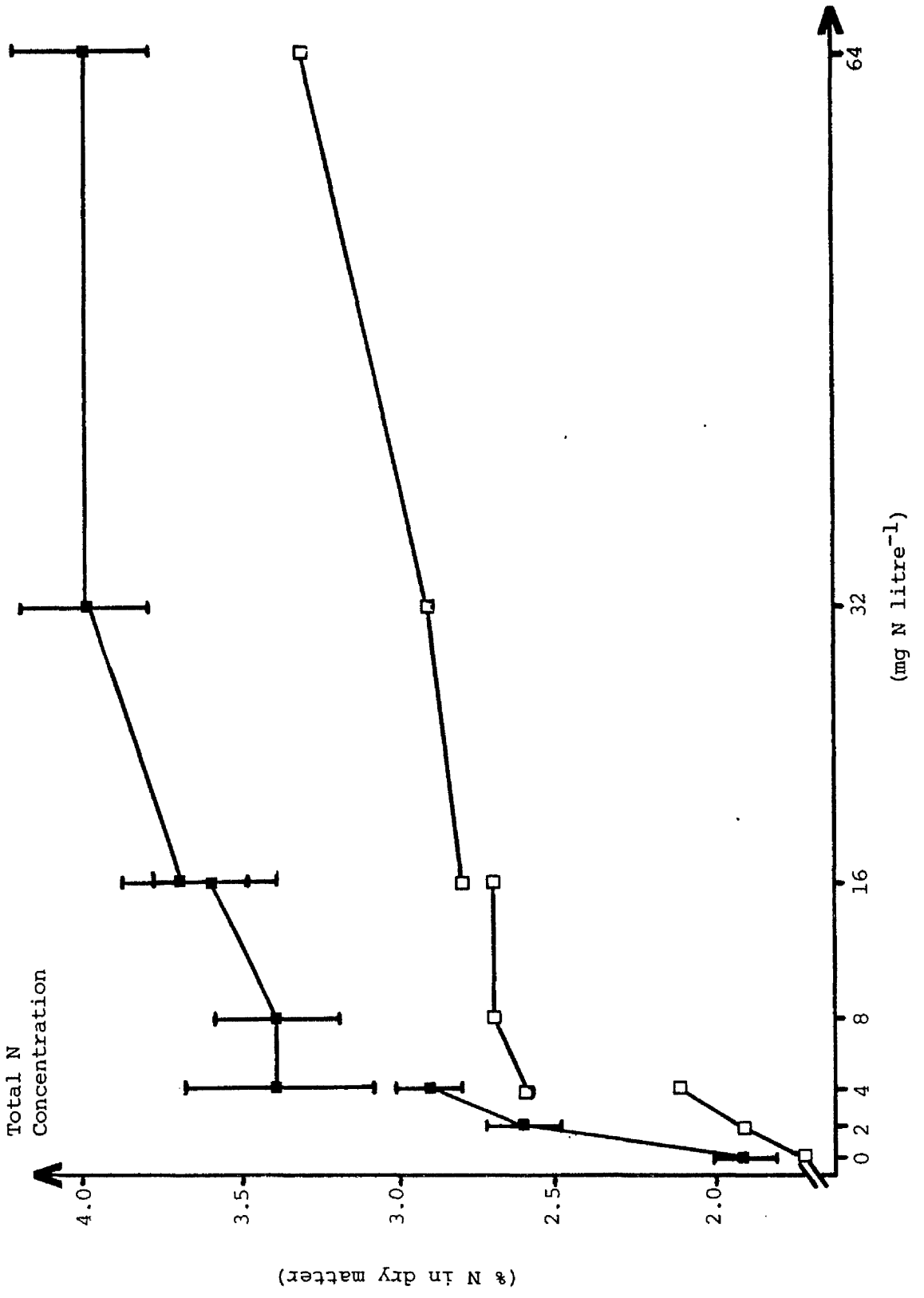

Total N
Concentration
(% N in dry matter)
4.0
3.5
3.0
2.5
2.0
0
2
4
8
16
32
64
(mg N litre^{-1})
Nitrate
Concentration
Supplied

FIGURE 36

Shoot and root tissue NO_3^- concentration versus NO_3^- concentration maintained in solution

Shoot tissue NO_3^- ■——■

Root tissue NO_3^- □——□

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means of shoot results
Error bars omitted from root results for clarity, but coefficients of variation were all < 11%
Composite data from 3 experiments

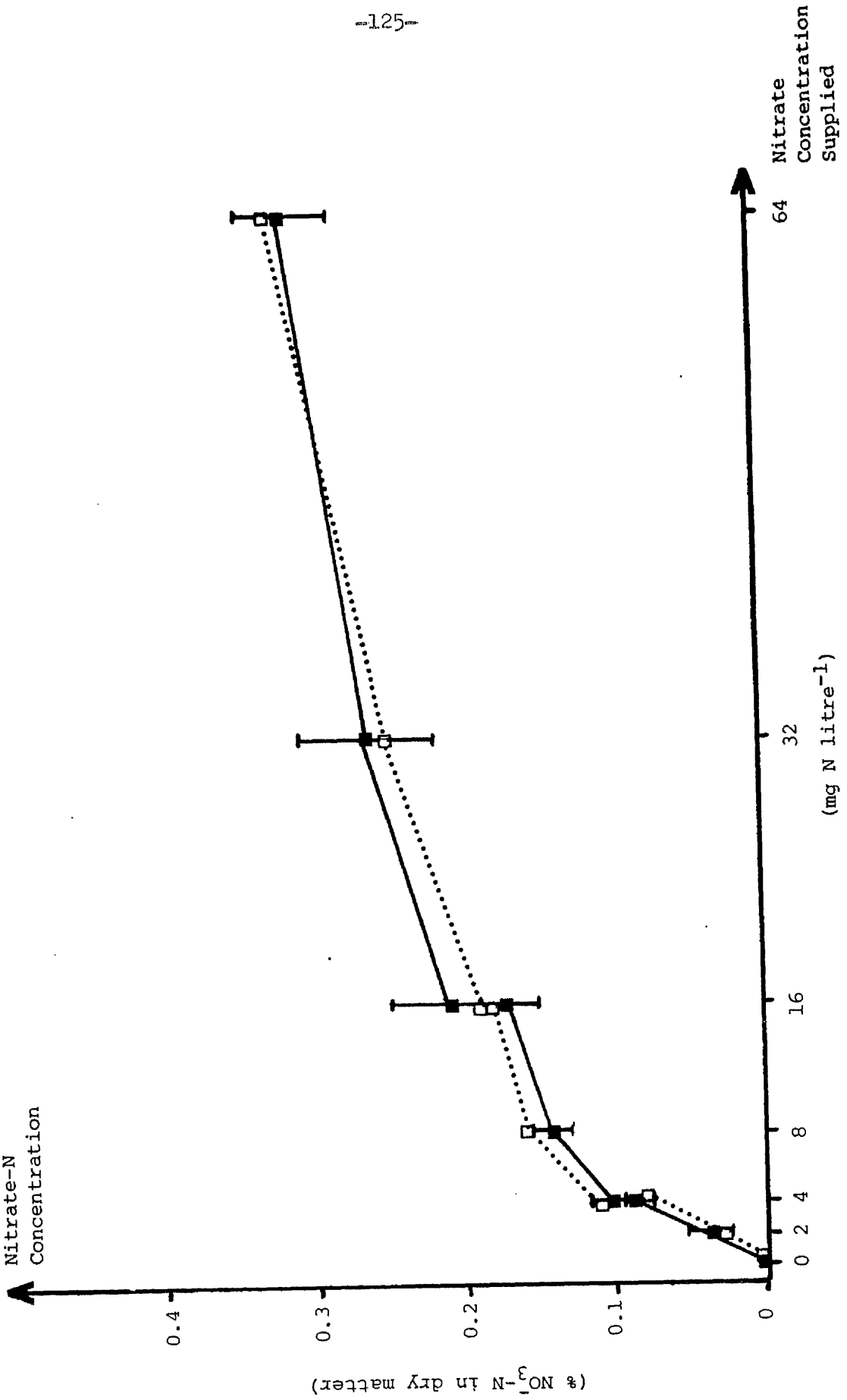

Nitrate-N Concentration
(% NO_3^--N in dry matter)
0.4
0.3
0.2
0.1
0
0 2 4 8 16 32 64
(mg N litre^{-1})
Nitrate Concentration Supplied

FIGURE 37

Shoot and root water soluble carbohydrate concentration versus NO_3^- concentration maintained in solution

Shoot carbohydrate ■——■

Root carbohydrate □——□

Each point represents the mean of 6 replicates
Error bars mark standard deviations of means of shoot results
Error bars omitted for clarity from root results, but coefficients of variation were all < 19%
Composite data from 3 experiments

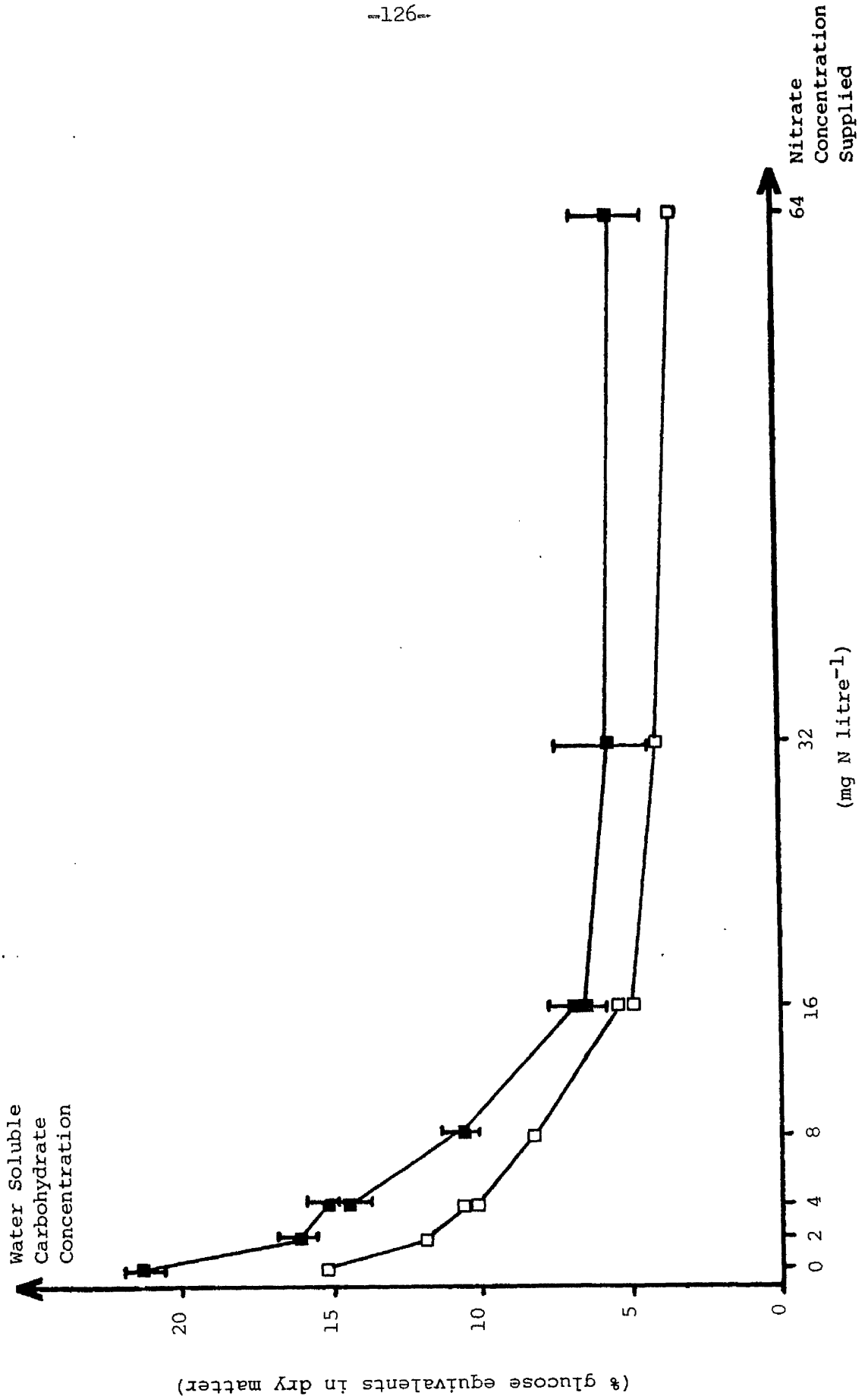

Water Soluble Carbohydrate Concentration
(% glucose equivalents in dry matter)
0
5
10
15
20
0 2 4 8 16 32 64
(mg N litre^{-1})
Nitrate Concentration Supplied

FIGURE 38

Shoot and root L-malate concentration versus NO_3^- concentration maintained in solution

Shoot L-malate ■——■

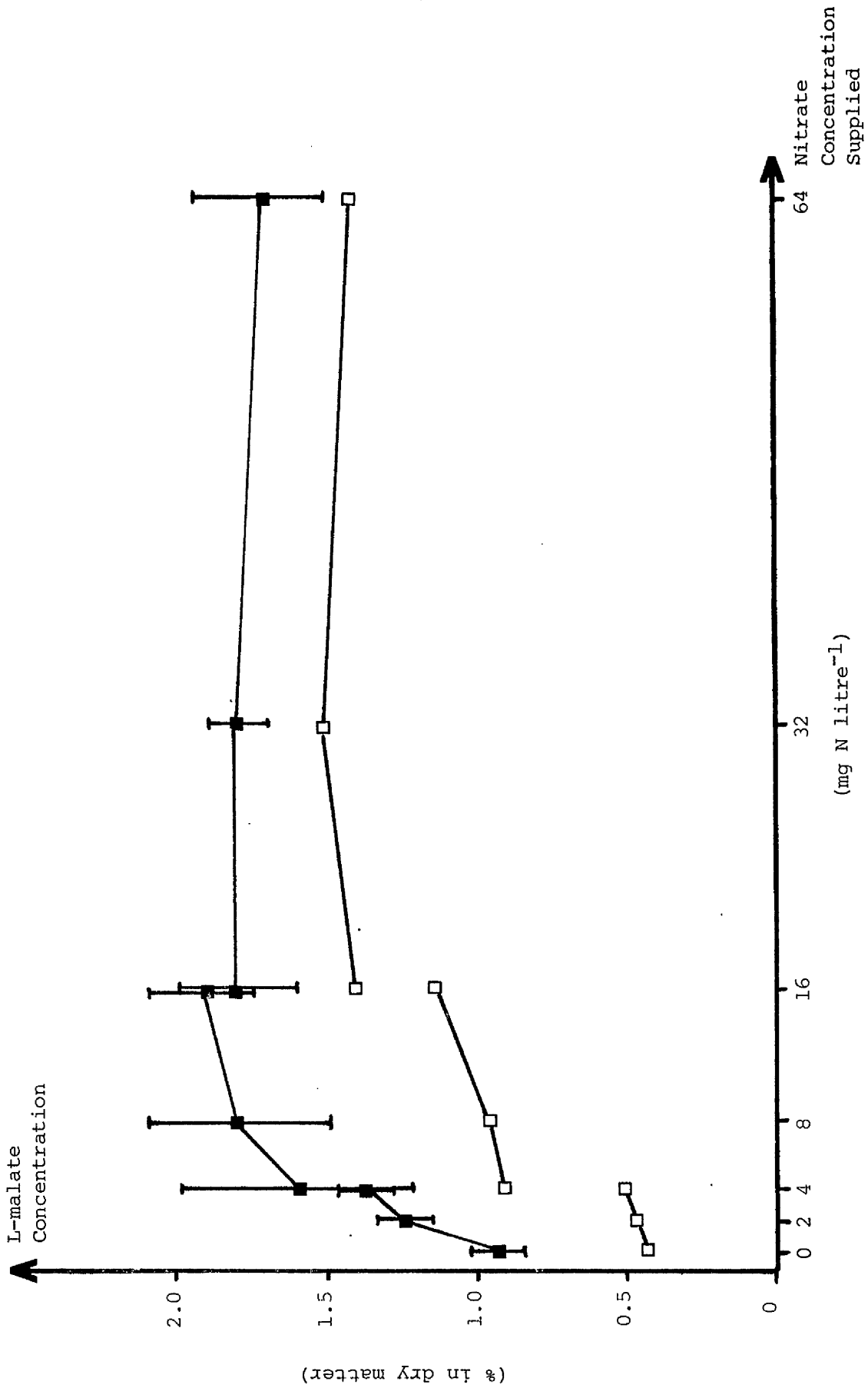
Root L-malate □——□

Each point represents the mean of 6 replicates

Error bars mark standard deviations of shoot results

Error bars omitted from root results for clarity, but coefficients of variation were all < 20%

Composite data from 3 experiments


L-malate Concentration
(% in dry matter)
2.0
1.5
1.0
0.5
0
0 2 4 8 16 32 64
Nitrate Concentration Supplied
(mg N litre^{-1})

4.6.4 Nitrogen form

Introduction

An earlier experiment (section 4.5.5) studied the effect on NRA of supplying barley plants with NH_4NO_3 and allowing nitrogen depletion to occur over a period of time. In the present experiment, the effect of form of nitrogen (NH_4^+, NH_4NO_3 or NO_3^-) supplied and maintained on growth, composition and NRA of barley plants was determined.

Experimental details

Barley plants were raised in the controlled environment room as described in section 4.6.2 with the following nitrogen forms and concentrations being maintained:

1) 20 ± 2mg $NH_4^+ - N\,l^{-1}$.

2) 10 ± 1mg $NH_4^+ - N\,l^{-1}$ and 10 ± 1mg $NO_3^- - N\,l^{-1}$.

3) 20 ± 2mg $NO_3^- - N\,l^{-1}$.

Temperature was $10 \pm 1^{o}C$, solution pH was kept in the range 6.8-6.3 and conductivity was kept at $2000 \pm 200\ \mu S\ cm^{-1}$.

Results

No differences in plant appearance were observed between treatments and the plants reached the 4th leaf stage after 21 days. They were then harvested and analysed as described earlier (section 4.6.2).

During the experiment, pH decreased in the NH_4^+ and NH_4NO_3 treatments and $Ca(OH)_2$ solution was added to keep the pH within the specified range. By contrast, pH increased in the NO_3^- treatment and H_2SO_4 was added to maintain pH. The reason for these pH changes was as explained previously (section 4.5.5).

The amounts of NO_3^- which had to be added to maintain initial concentrations were:

NH_4^+ treatment : 785mg NH_4^+ - N

NH_4NO_3 treatment : 598mg NH_4^+ - N and 80mg NO_3^- - N

NO_3^- treatment : 615mg NO_3^- - N

Preferential NH_4^+ utilization was apparent from the amounts of NH_4^+ and NO_3^- which had to be added to the NH_4NO_3 treatment. More NH_4^+ than NO_3^- also appeared to be utilized when they were supplied individually. These results are in general agreement with those of Breteler and Smit (1974).

Fresh and dry weights and % dry matter

TABLE 18: Effect of form of nitrogen supplied on fresh and dry weights and % dry matter

	Form of nitrogen		
	NH_4^+	NH_4NO_3	NO_3^-
Shoot fresh weight (g)	24_a	25_a	20_b
Shoot dry weight (g)	2.4_a	2.6_a	2.1_b
Shoot % dry matter	10.4_a	10.6_a	10.8_a
Root dry weight (g)	0.60_a	0.66_b	0.63_b

Results are means of 6 replicates.

Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Shoot fresh and dry weights were higher in plants fed NH_4^+ and NH_4NO_3 than in those supplied with NO_3^- (Table 18). This may have been due to greater nitrogen uptake and conversion to protein in NH_4^+- and NH_4NO_3-fed plants than in those fed NO_3^-. Energy saved by NH_4^+

utilisation, in preference to NO_3^-, could perhaps have been used to enhance growth. Higher dry weights were also observed in NH_4^+-fed plants by Smith & Rice (1983).

No marked differences were found in shoot % dry matter, or in dry weights of roots recovered, between treatments (Table 18).

NRA_e, NRA_i and NAC ratios

TABLE 19: Effect of form of N supplied on shoot and root NRA_e, NRA_i and NAC ratios

	Form of nitrogen		
	NH_4^+	NH_4NO_3	NO_3^-
Shoot NRA_e (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.07_a	0.87_b	1.5_c
Shoot NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.10_a	1.01_b	1.55_c
Shoot NAC ratio	-	1.2_a	1.06_b
Root NRA_e (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.06_a	0.28_b	0.84_c
Root NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.05_a	0.34_b	0.94_c
Root NAC ratio	-	1.2_a	1.1_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Shoot and root NRA_e and NRA_i levels were very low in plants fed NH_4^+, and highest in plants fed NO_3^- (Table 19). Plants supplied only with NH_4^+ have no need for NR and there was supposedly no NO_3^- available to induce NR. However, traces of NO_3^- were detected in the "all NH_4^+" nutrient solution. This NO_3^- was possibly produced from NH_4^+

by nitrifying bacteria and could have induced the very low NRA detected in NH_4^+ fed plants, an effect also seen by Minotti et al., (1969), Ferguson & Knypl (1974) and Fair et al., (1974). By contrast, no NRA was detected in NH_4^+ grown plants by Afridi & Hewitt (1964) and Weismann (1972).

NRA levels were intermediate in NH_4NO_3 fed plants due to NH_4^+ inhibition or repression of NR or to only small amounts of NO_3^- being taken up (see also section 4.5.5). Smith & Rice (1983) also noted that NRA was intermediate in NH_4NO_3 fed plants.

NAC ratios were not markedly different between NH_4NO_3- and NO_3^--fed plants, even though NRA_e and NRA_i levels differed widely (Table 19). It was not meaningful to calculate the NAC ratio for NH_4^+-fed plants since NRA_e and NRA_i were very low.

Dry matter analyses

Table 20 shows results of shoot and root dry matter analyses.

TABLE 20: Effect of form of N supplied on shoot and root total N, NO_3^--N, water soluble carbohydrate and L-malate contents

	Form of nitrogen		
	NH_4^+	NH_4NO_3	NO_3^-
Shoot total N (% in dry matter)	4.7_a	4.5_a	4.1_b
Shoot NO_3^--N (% in dry matter	$<0.01_a$	0.06_b	0.16_c
Shoot water soluble carbohydrate (% glucose equivalents in dry matter)	4.9_a	5.0_a	5.7_b
Shoot L-malate (% in dry matter)	1.6_a	1.9_b	2.3_c
Root total N (% in dry matter)	3.2_a	3.2_a	2.9_b
Root NO_3^--N (% in dry matter)	$<0.01_a$	0.08_b	0.16_c
Root water soluble carbohydrate (% glucose equivalents in dry matter)	2.1_a	2.9_b	3.1_c
Root L-malate (% in dry matter)	0.7_a	0.84_a	1.0_b

Results are means of 6 replicates. (Cont....)

Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Total N contents of shoots and roots of plants fed NH_4^+ or NH_4NO_3 were higher than of those supplied with NO_3^- only (Table 20), confirming that there was a greater uptake and incorporation of nitrogen by NH_4^+- and NH_4NO_3-fed plants than by those supplied with only NO_3^-. Plants supplied with NH_4^+ have often been found to have higher total N concentrations than NO_3^--fed plants (Harada et al., 1968 and Blair et al., 1970).

There was a trace of NO_3^- in shoots and roots of plants fed NH_4^+, while those supplied with NO_3^- had the highest amounts of tissue NO_3^- (Table 20). Traces of NO_3^- in NH_4^+ fed plants are assumed to be due to the plants having taken up NO_3^- produced by nitrifying bacteria and to the very low NRA of these plants having incompletely reduced it.

Water soluble carbohydrate concentrations in shoots and roots of NH_4^+- and NH_4NO_3-fed plants were less than in NO_3^--fed plants (Table 20), possibly as a result of the greater demand for carbohydrates to combine with NH_4^+ to produce more amino acids and proteins in roots of NH_4^+ and NH_4NO_3 fed plants.

L-malate contents were lowest in shoots and roots of NH_4^+-fed plants, and highest in NO_3^--fed plants (Table 20). It has been proposed that L-malate is produced in stoichiometric amounts when NO_3^- is reduced in leaves (Ben-Zioni et al., 1970). It has been further hypothesized that malate moves to the roots (as K^+ malate), where it is decarboxylated and the resulting $KHCO_3$ then exchanges for NO_3^- i.e. NO_3^- uptake occurs (Ben-Zioni et al., 1971). If these hypotheses are correct, it is likely that those plant tissues reducing more NO_3^- will

have higher L-malate contents, as found here. Substitution of NH_4^+ for NO_3^-, nutrition was shown to reduce the accumulation of malate and other carboxylates (Breteler & Smit, 1974).

Conclusions

The following conclusions were reached in the study of the effects of form of nitrogen supplied on growth, composition and NRA of barley plants:

a) NH_4^+ was utilised in preference to NO_3^- by plants supplied with NH_4NO_3. More NH_4^+ than NO_3^- was probably utilised by plants when these nutrients were supplied individually.

b) Shoot fresh and dry weights were greater in plants fed NH_4^+ and NH_4NO_3 than in those fed NO_3^-.

c) Shoot and root NRA_e and NRA_i were highest in plants fed NO_3^-, intermediate in plants fed NH_4NO_3 and very low in plants supplied only with NH_4^+. The very low NRA of NH_4^+ fed plants was probably induced by traces of NO_3^- produced by NH_4^+ oxidising bacteria. NAC ratios could not be calculated for NH_4^+-fed plants, but NAC ratios of NH_4NO_3 and NO_3^--fed plants were not very different.

d) Shoot and root total N contents were greater, and soluble carbohydrate concentrations were lower, in plants fed NH_4^+ or NH_4NO_3 than in those fed NO_3^-. Shoot and root tissue NO_3^- concentrations and L-malate concentrations were highest in plants fed NO_3^- and lowest in NH_4^+-fed plants.

4.6.5 Nutrient solution pH

Introduction

Although intracellular pH is not altered much by external pH (Raven & Smith, 1980), variations in external pH may alter rates of NO_3^- uptake by plants and hence affect NRA. The effect of various nutrient solution pH values (pH 4.0, 5.3 and 6.5) on growth, composition and NRA of barley plants were therefore investigated.

Experimental details

Plants were grown in the controlled environment room as described in section 4.6.2 using nutrient solutions maintained at pH 4.0 $\pm$ 0.2, 5.3 $\pm$ 0.2 and 6.5 $\pm$ 0.2. The nitrogen concentration in all three treatments was maintained at 8 $\pm$ 1mg NO_3^- - N l^{-1} . Solution conductivity was kept at 2000 $\pm$ 200 $\mu S\ cm^{-1}$ and temperature was 10 $\pm$ 1^oC.

Results

Plants in all three treatments were similar in appearance and reached the 4th leaf stage 21 days after starting the treatments. They were then harvested and analysed as described previously (section 4.6.2).

During the experiment, pH tended to increase in all the treatments and H_2SO_4 was added to keep pH within the specified ranges.

The amounts of NO_3^- which had to be added to maintain initial concentrations were:

pH 4.0 treatment : 684mg NO_3^- - N

pH 5.3 treatment : 617mg NO_3^- - N

pH 6.5 treatment : 548mg NO_3^- - N

More NO_3^- had to be added to the lower, than to the higher, pH treatments to maintain initial concentrations, and it is probable that more NO_3^- was taken up by plants growing at lower pH, a phenomenon also noted by Rao & Rains (1976) and Iken & Marcus-Wyner (1984).

Fresh and dry weights and % dry matter

TABLE 21: Effect of pH of nutrient solution supplied on fresh and dry weights and % dry matter

	Nutrient solution pH		
	4.0	5.3	6.5
Shoot fresh weight (g)	26_a	27_a	21_b
Shoot dry weight (g)	3.0_a	3.0_a	2.7_b
Shoot % dry matter	11.7_a	11.1_a	12.9_b
Root dry weight (g)	0.8_a	0.9_a	0.8_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Shoot fresh and dry weights were higher in plants grown at pH 4.0 and 5.3 than at pH 6.5 (Table 21), possibly as a result of greater NO_3^- uptake, conversion to protein and production of more growth in plants grown at lower pH.

% dry matter was lower in shoots of plants grown at pH 4.0 and 5.3 than in those grown at pH 6.5. No statistically significant differences in dry weights of roots recovered were detected (Table 21).

NRA_e, NRA_i and NAC ratios

TABLE 22: Effect of pH of nutrient solution supplied on shoot and root NRA_e, NRA_i and NAC ratios

	Nutrient solution pH		
	4.0	5.3	6.5
Shoot NRA_e (μmol $NO_2^-g^{-1}$ fresh weight h^{-1})	1.45_a	1.4_a	1.2_b
Shoot NRA_i (μmol $NO_2^-g^{-1}$ fresh weight h^{-1})	1.66_a	1.55_b	1.41_c
Shoot NAC ratio	1.15_a	1.2_a	1.2_a
Root NRA_e (μmol $NO_2^-g^{-1}$ fresh weight h^{-1})	0.8_a	0.7_a	0.7_a
Root NRA_i (μmol $NO_2^-g^{-1}$ fresh weight h^{-1})	0.87_a	0.74_b	0.70_b
Root NAC ratio	1.2_a	1.1_a	1.1_a

Results are means of 6 replicates.

Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

NRA_e and NRA_i were highest in shoots of plants grown at pH 4.0 and lowest in those grown at pH 6.5 (Table 22). Higher NRA_e and NRA_i in shoots of plants grown at lower pH may be the result of greater NO_3^- uptake and greater induction of NR. Iken & Marcus-Wyner (1984) observed that tomato shoot NRA was higher in plants grown at pH 4.5 than at pH 6.5. Trends in root NRA_e and NRA_i were similar to those in shoots, although differences were less marked (Table 22).

There were no marked differences in shoot or root NAC ratios between treatments, although NRA_e and NRA_i values differed (Table 22).

Dry matter analyses

Table 23 shows results of shoot and root dry matter analyses.

TABLE 23: Effect of pH of nutrient solution supplied on shoot and root total N, NO_3^--N, water soluble carbohydrate and L-malate contents.

	Nutrient solution pH		
	4.0	5.3	6.5
Shoot total N (% in dry matter)	3.6_a	3.5_a	3.7_a
Shoot NO_3^--N (% in dry matter)	0.25_a	0.18_b	0.14_c
Shoot water soluble carbohydrate (% glucose equivalents in dry matter)	11_a	10_a	12_b
Shoot L-malate (% in dry matter)	1.8_a	1.7_a	1.6_a
Root total N (% in dry matter)	2.6_a	2.9_b	2.8_{ab}
Root NO_3^--N (% in dry matter)	0.24_a	0.20_a	0.13_b
Root water soluble carbohydrate (% glucose equivalents in dry matter)	9_a	9_a	10_a
Root L-malate (% in dry matter)	1.3_a	1.1_b	1.0_b

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

No marked differences in shoot or root total N contents were detected between the treatments (Table 23).

Tissue NO_3^- contents were highest in shoots and roots of plants grown at pH 4.0, and lowest in those grown at pH 6.5 (Table 23). This higher NO_3^- content of plants grown at lower pH may have caused induction of the higher NRA observed in these plants.

There were no marked differences in shoot or root water soluble

carbohydrate or L-malate contents between the treatments (Table 23).

Conclusions

The conclusions reached in the study of the effects of nutrient solution pH supplied on growth, composition and NRA of barley plants were:

a) More NO_3^- was taken up by plants from nutrient solution at pH 4.0 than at pH 6.5.

b) Shoot fresh and dry weights were higher in plants grown with nutrient solutions of pH 4.0 and 5.3 than in those grown at pH 6.5. Shoot % dry matter was lower in plants grown at pH 4.0 and 5.3 than in those grown at pH 6.5.

c) Shoot and root NRA_e and NRA_i were greater in plants grown at pH 4.0 than in those grown at pH 6.5. The pH treatments used had no effect on shoot or root NAC ratios.

d) Nutrient solution pH had no marked effect on shoot or root total N, water soluble carbohydrate or L-malate concentrations. Shoot and root tissue NO_3^- concentrations were greater in plants grown at pH 4.0 than in those grown at pH 6.5.

4.6.6 Root temperature

Introduction

Experiments performed in growth cabinets, using high light intensities and high temperatures (often 25^{o}C) are far removed from conditions experienced by field crops. Also, temperatures experienced by above ground portions of plants grown in the field can be markedly different from those experienced by roots. In autumn/early winter, soil temperatures can be considerably higher than average air

temperatures; while in spring, average air temperatures can be considerably higher than soil temperatures, due to differential rates of heating and cooling of soil and air, (Clarkson & Warner, 1979 and Deane-Drummond et al., 1980). Accordingly, the effects of different root temperatures (5, 10 and 15^{o}C) with a common, relatively low air temperature (10^{o}C) on growth, composition and NRA of barley plants were investigated.

Experimental details

Plants were raised in the controlled environment room as described in section 4.6.2 with root temperatures of 5 $\pm$ 1, 10 $\pm$ 1 and 15 $\pm$ 1^{o}C and a 10 $\pm$ 1^{o}C air temperature. The nitrogen concentration in the nutrient solution was maintained at 8 $\pm$ 1mg NO_3^- - N l^{-1}. pH was kept in the range 6.8-6.3 and solution conductivity was kept at 2000 $\pm$ 200 $\mu S\,cm^{-1}$.

Results

All the plants were harvested when the plants in the 10^{o}C treatment reached the 4th leaf stage (21 days after starting the treatments). At this time, plants in the 15^{o}C treatment were slightly more developed, having reached the 4th leaf stage on day 18, while those in the 5^{o}C treatment had not yet reached the 4th leaf stage. The plant material was then analysed as described previously (section 4.6.2).

Solution pH tended to increase in all the treatments and H_2SO_4 was added to keep pH in the range 6.8-6.3.

The amounts of NO_3^- which had to be added to maintain the initial concentrations were:

5^oC treatment : 479mg NO_3^- - N

10^oC treatment : 556mg NO_3^- - N

15^oC treatment : 604mg NO_3^- - N

More NO_3^- had to be added to higher root temperature treatments and therefore more NO_3^- was probably taken up by plants grown at higher, than at lower root temperatures.

Fresh and dry weights and % dry matter

TABLE 24: Effect of root temperature on fresh and dry weights and % dry matter

	Root temperature (^oC)		
	5	10	15
Shoot fresh weight (g)	16_a	19_{ab}	23.5_b
Shoot dry weight (g)	2.1_a	2.5_a	2.7_a
Shoot % dry matter	12.8_a	12.9_{ab}	11.7_b
Root dry weight (g)	0.5_a	0.6_a	0.6_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Shoot fresh weights were lowest in plants grown with a root temperature of 5^oC and highest at 15^oC (Table 24). A similar trend was found in shoot dry weights, but differences were not statistically significant. These trends may be due to greater nutrient uptake by roots from higher temperature solutions supporting more growth.

Shoot % dry matter was higher in plants grown at root temperatures

of 5°C than at 15°C. No differences in dry weights of roots recovered were detected (Table 24).

NRA_e, NRA_i and NAC ratios

TABLE 25: Effect of root temperature on shoot and root NRA_e, NRA_i and NAC values

	Root temperature (°C)		
	5	10	15
Shoot NRA_e (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	1.2_a	1.1_a	1.2_a
Shoot NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	1.5_a	1.4_a	1.4_a
Shoot NAC ratio	1.3_a	1.3_a	1.2_a
Root NRA_e (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.8_a	0.6_b	0.5_c
Root NRA_i (μmol $NO_2^- g^{-1}$ fresh weight h^{-1})	0.8_a	0.7_b	0.6_b
Root NAC ratio	1.1_a	1.1_a	1.2_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

No statistically significant differences in shoot NRA_e, NRA_i or NAC ratio were noted between the treatments (Table 25). By contrast, root NRA_e and NRA_i were highest in plants grown with a root temperature of 5°C and lowest in those grown at 15°C (Table 25). NRA is governed by relative rates of NR synthesis or activation and of degradation or inactivation (Travis et al., 1969). At lower root

temperatures, rates of both synthesis/activation and of degradation/inactivation will be lower than at higher temperatures, as a consequence of the influence of temperature on enzyme kinetics. However, if the rate of synthesis/activation decreases less than the rate of degradation/inactivation decreases, the concentration of NR, and hence NRA, will be greater at lower temperatures. Gosselin et al., (1984) showed that root NRA of tomatoes increased as root temperature decreased from 30°C to 12°C while Blahova, (1982) observed that transfer of cucumber plants from a temperature of 2°C to 20°C caused a decline in NRA.

No statistically significant differences in root NAC ratio were found between the treatments, although magnitudes of NRA_e and NRA_i were different (Table 25).

Dry matter analyses

Table 26 shows results of shoot and root dry matter analyses.

TABLE 26: Effect of root temperature on shoot and root total N, NO_3^--N, water soluble carbohydrate and L-malate contents

	Root temperature (°C)		
	5	10	15
Shoot total N (% in dry matter)	3.4_a	3.6_b	3.7_{ab}
Shoot NO_3^--N (% in dry matter)	0.14_a	0.15_{ab}	0.19_b
Shoot water soluble carbohydrate (% glucose equivalents in dry matter)	14_a	15_a	14_a
Shoot L-malate (% in dry matter)	1.7_a	1.7_a	1.5_a
Root total N (% in dry matter)	2.4_a	2.7_{ab}	2.8_b
Root NO_3^--N (% in dry matter)	0.14_a	0.20_b	0.24_b
Root water soluble carbohydrate (% glucose equivalents in dry matter)	16_a	13_b	11.5_b
Root L-malate (% in dry matter)	1.4_a	1.2_b	0.9_c

Results are means of 6 replicates.

Results followed by the same letter(s) (compared horizontally) are not

significantly different at the 5% level.

Shoot and root total N contents tended to be higher in plants grown with higher root temperatures (Table 26).

Shoot and root tissue NO_3^- contents were lowest in plants grown with a 5^oC root temperature and highest in those grown with a 15^oC root temperature (Table 26).

No statistically significant differences in shoot water soluble carbohydrate or shoot L-malate contents were found between the treatments, but root water soluble carbohydrate and L-malate concentrations were highest in plants grown with a root temperature of 5^oC and lowest in those grown at 15^oC (Table 26). Similar trends were also observed by Clarkson et al., (1974) and Deane-Drummond et al., (1980).

Conclusions

The conclusions reached in the study of the effects of root temperature on growth, composition and NRA of barley plants were:

a) More NO_3^- was taken up by plants as the temperature of the nutrient solution increased.

b) Shoot fresh and dry weights increased as root temperature increased. Shoot % dry matter decreased as root temperature increased.

c) There were no differences in shoot NRA_e, NRA_i or NAC ratio between plants grown with root temperatures of 5, 10 or 15^oC. By contrast, root NRA_e and NRA_i values were greatest at the lowest root temperature tested. There were no differences in root NAC ratio between the treatments.

d) Shoot and root total N and NO_3^--N concentrations tended to increase as the root temperature which the plants were grown at increased. Root temperature had no effect on shoot water soluble carbohydrate

or L-malate concentrations, but root carbohydrate and malate contents were greater in plants grown at lower, than at higher, root temperatures.

4.6.7 Shoot temperature

Introduction

The effect on growth, composition and NRA of barley plants of differential shoot/root temperatures studied earlier (section 4.6.6) was further investigated, by growing barley plants at different air temperatures (6 and 17°C), while root temperatures were kept at 10°C.

Experimental details

The controlled environment room described in section 4.6.2 was reorganized into a large, cooled area and a small, enclosed heated area; and one treatment was established in each area. The two air temperatures used were 6 ± 2 and 17 ± 3°C, while root temperature was 10 ± 1°C in both cases. Nitrogen concentrations in nutrient solutions were maintained at 8 ± 1mg NO_3^- - Nl^{-1}, pH was maintained in the range 6.8-6.3 and solution conductivity was kept at 2000 ± 200 μScm^{-1}.

Results

Plants were grown under these conditions for 21 days, by which time the plants in the 17°C treatment had developed to the 5th leaf stage, while plants in the 6°C treatment had not quite reached the 4th leaf stage. The plants were then harvested and analysed as described previously (section 4.6.2).

pH tended to increase in both treatments and H_2SO_4 was added to keep pH within the range 6.8-6.3.

The amounts of NO_3^- which had to be added to maintain initial

concentrations were:

$6^{o}C$ treatment : 473mg NO_3^- - N

$17^{o}C$ treatment : 691mg NO_3^- - N

More NO_3^- had to be added to the solution supplied to the plants grown at the higher air temperature, indicating that more NO_3^- was utilised by these plants than by those grown with the lower shoot temperature.

Fresh and dry weights and % dry matter

TABLE 27: Effect of shoot temperature on fresh and dry weights and % dry matter

	Shoot temperature (^{o}C)	
	6	17
Shoot fresh weight (g)	18_a	26_b
Shoot dry weight (g)	2.3_a	3.0_b
Shoot % dry matter	12.7_a	11.5_b
Root dry weight (g)	0.55_a	0.55_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Fresh and dry weights were higher in shoots of plants grown at an air temperature of $17^{o}C$ than in those grown at an air temperature of $6^{o}C$ (Table 27). The greater amount of NO_3^- taken up may have produced more protein and growth in the higher shoot temperature treatment plants.

% dry matter was higher in shoots of plants grown with an air temperature of $6^{o}C$, than in those grown at $17^{o}C$. No differences in dry weight of roots recovered were found (Table 27).

NRA_e, NRA_i and NAC ratios

TABLE 28: Effect of shoot temperature on shoot and root NRA_e, NRA_i and NAC ratios

	Shoot temperature (^oC)	
	6	17
Shoot NRA_e ($\mu mol\ NO_2^- g^{-1}$ fresh weight h^{-1})	1.21_a	0.92_b
Shoot NRA_i ($\mu mol\ NO_2^- g^{-1}$ fresh weight h^{-1})	1.38_a	1.09_b
Shoot NAC ratio	1.1_a	1.2_b
Root NRA_e ($\mu mol\ NO_2^- g^{-1}$ fresh weight h^{-1})	0.64_a	0.74_b
Root NRA_i ($\mu mol\ NO_2^- g^{-1}$ fresh weight h^{-1})	0.66_a	0.79_b
Root NAC ratio	1.0_a	1.1_a

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

NRA_e and NRA_i were higher in shoots of plants grown at an air temperature of 6^oC than in those grown at an air temperature of 17^oC (Table 28). This was probably due to differential reduction in rates of NR synthesis/activation and degradation/inactivation at low temperature (see section 4.6.6). Other workers have also found shoot NRA of various species to be greater at lower, than at higher, temperatures (Harmer & Lee, 1981; Harris & Whittington, 1983 and Moroz et al., 1984).

In contrast to shoot NRA results, root NRA_e and NRA_i values were

slightly greater in plants grown with an air temperature of 17°C than in those grown at 6°C (Table 28).

Shoot and root NAC ratios were not markedly different between the two treatments (Table 28).

Dry matter analyses

Table 29 shows results of shoot and root dry matter analyses.

TABLE 29: Effect of shoot temperature on shoot & root total N, NO_3^--N, water soluble carbohydrate and L-malate contents

	Shoot temperature (°C)	
	6	17
Shoot total N (% in dry matter)	3.4_a	4.0_b
Shoot NO_3^--N (% in dry matter)	0.13_a	0.21_b
Shoot water soluble carbohydrate (% glucose equivalents in dry matter)	18_a	14_b
Shoot L-malate (% in dry matter)	1.8_a	1.5_b
Root total N (% in dry matter)	2.8_a	2.8_a
Root NO_3^--N (% in dry matter)	0.15_a	0.22_b
Root water soluble carbohydrate (% glucose equivalents in dry matter)	15.5_a	12_b
Root L-malate (% in dry matter)	1.3_a	1.2_b

Results are means of 6 replicates.
Results followed by the same letter(s) (compared horizontally) are not significantly different at the 5% level.

Total N content was greater in shoots, but not in roots, of plants grown at an air temperature of 17°C than in those grown at an air temperature of 6°C (Table 29). This confirms that more NO_3^- was taken up by plants grown at the higher, than at the lower air temperature.

Shoot and root tissue NO_3^- contents were greater in plants

grown at an air temperature of 17°C than in those grown at 6°C (Table 29).

Water soluble carbohydrate and L-malate concentrations were higher in shoots and roots of plants grown at an air temperature of 6°C than at 17°C (Table 29).

Conclusions

The following conclusions were reached in the study of the effects of shoot temperature on growth, composition and NRA of barley plants:

a) More NO_3^- was taken up from solutions at 10°C by plants grown with air temperatures of 17°C than by those grown with air temperatures of 6°C.

b) Shoot fresh and dry weights were higher, and % dry matter was lower in plants grown with an air temperature of 17°C than in those grown with an air temperature of 6°C.

c) Shoot NRA_e and NRA_i were greater in plants grown at the lower, than at the higher, air temperature. In contrast, root NRA_e and NRA_i were slightly greater in plants grown at the higher, than at the lower, air temperature. There were no marked differences in shoot or root NAC ratios between treatments.

d) Shoot total N and shoot and root NO_3^--N contents were higher in plants grown with the higher, than in those grown with the lower, air temperature, but there were no differences in root total N contents between treatments. Shoot and root water soluble carbohydrate and L-malate contents were greater in plants grown with the lower, than with the higher, air temperature.

5. GENERAL DISCUSSION

Development of improved methods for predicting optimum N fertilizer rates and timings is widely recognized as a pressing and outstanding need in modern agriculture (Royal Society, 1983). Improved prediction techniques must be sought to both increase crop production and to avoid waste of expensively produced nitrogen fertilizer. In addition, water pollution must be reduced by using carefully determined N fertilizer rates and timings to minimise N leaching and run off. At present, typical recovery rates of N fertilizer are only 40-60% by cereal crops, and 60-75% by herbage crops (Holmes, 1979).

Factors influencing crop N requirements include crop yield and quality desired, previous cropping and manuring, effects of winter leaching and summer drought. The current MAFF system of N fertilizer application rate recommendations for arable crops is based on consideration of these factors (Royal Society, 1983). The ADAS N index system (Halley, 1982) gives only a general guide. It is divided into 3 classes: N index 0 - minimal soil N reserves, N index 1 - medium N reserves and N index 2 - large N reserves (ICI, 1983). Soil N analysis results are used successfully in continental Europe as a predictive tool. In the U.K., high variability of results, and the vagaries of the British maritime climate (Simpson, 1983) have made this method grossly inaccurate, to the extent of being even less reliable than the N-index system (ICI, 1983).

There is clearly a need for a quick, reliable test for predicting plant and/or soil N status and also crop N fertilizer

requirements. Many workers have tried various approaches, including soil and plant tests, mathematical modelling and biochemical tests.

In general, methods of soil N analysis, including incubation techniques, have not been successful as predictors of N fertilizer rates (Mengel & Kirkby, 1982; Batey, 1982 and Royal Society, 1983). The problem with soil N analysis is that it does not take account of the additional amount of N which is produced by mineralisation and which becomes available to the crop over the growing period after the soil test. It seems unlikely that a practical method of soil N analysis will be found which will act as an indicator of crop fertilizer N requirements due to the above problem, and to very variable results caused by unpredictable variations in weather and crop response between sites and from year to year.

The use of mathematical/computer modelling has been proposed to provide a better approach than soil tests as a predictive tool (Barraclough, 1979 and Simpson, 1983). However, the technique is still in its infancy and much more work will be required to model for the gamut of environmental and other factors (e.g. rainfall, soil type, past cropping and manuring, soil organic matter, temperature, rate of mineralisation, etc.) which greatly influence crop N uptake/demand. The large number of parameters involved, with their associated prediction errors and uncertainties may render these models unworkable/unwieldy and commercially unattractive.

Many workers have also tried to use plant N analysis to determine crop N status and predict N fertilizer requirements, although results in general have been very disappointing. Total N analysis of dried plant material has been the most common approach, but most

workers have found the technique to be of little use as a predictive tool (Bar-Akiva, 1970; Verstraeten & Vlassak, 1981 and Batey, 1982). The main problem with total N analysis is in taking standard leaf samples, since total N can vary greatly in tissues of different age. Also, the total N content of a plant represents the sum of biochemically active and inactive nitrogen. Hence, total N is unlikely to be suitable as a predictor of the true dynamic N status of the plant tested.

Another common plant N analytical technique uses the NO_3^- concentration of tissue, or of fresh sap, as a predictive measure of plant N status. However, most workers have found tissue NO_3^- analysis to be as equally unsuitable as total N analysis (Bar-Akiva, 1970; Verstraeten & Vlassak, 1981 and Mengel & Kirkby, 1982). The drawback of NO_3^- analysis is that NO_3^- contents of plants can be highly variable between tissues and over short periods of time. Also, NO_3^- is readily accumulated in large amounts in vacuoles beyond the optimum level required by the plant.

Similar problems have been encountered by other workers attempting to use tissue amine, amide or other nitrogenous fractions (Jungk & Wehman, 1978) as predictive measures of plant N status. None of these above methods of plant N analysis have been found to be adequate for use as a predictive tool for optimum fertilizer N rate determinations over a wide range of conditions (from site to site and from year to year).

Combined soil N and plant N analyses have also been tested as predictors of crop N needs with some success (Wehrman, 1982), but not to the extent of being commercially viable (Oosterhuis & Bate, 1983). The main problem with combined soil/plant tests is

the difficulty in determining "critical" values of soil and plant N concentrations required.

The general lack of success of simple soil, plant and combined soil/plant N analyses as predictive tools for determining crop N status and optimum fertilizer N rates has led some workers to explore potentially more complex avenues, such as the use of mathematical modelling (already mentioned) and, increasingly, the use of biochemical tests on living plant tissues as predictive tools.

Biochemical tests are based on determinations of the activity of the enzyme nitrate reductase (NR) which catalyses the first step in the incorporation of NO_3^- into plant protein. It has been suggested that NRA differentiates between metabolically active and inactive N fractions (Bar-Akiva, 1967 and Bar-Akiva, 1970), and workers have found variations in NRA to be more sensitive to changes in soil and plant N levels than analyses of tissue total N or NO_3^--N levels (Johnson, 1976 and Oosterhuis & Bate, 1983).

Many workers have proposed NRA to be correlated with the yield of crops as varied as citrus (Bar-Akiva, 1967), Lolium (Bowerman & Goodman, 1971), wheat (Eilrich & Hageman, 1973) and grain amaranth (Ramamurthy-Naidu et al., 1982). In addition, correlations between NRA and grain % protein (Nair & Abrol, 1982), free amino N (Friedrich et al., 1977) and growth rate of Laminaria (Davison et al., 1984) have been found.

An early proposed application of NRA measurements was as a selection tool for determining high yield or high protein cultivars in crop breeding programmes (Hageman et al., 1961 and Dalling & Loyn, 1977). High seedling NRA has been proposed to indicate a

cultivar's potential for metabolizing large amounts of NO_3^-, and hence for producing greater yields and/or higher protein contents than low NRA varieties (Austin et al., 1978 and Baer & Collet, 1981). A further suggested application of NRA measurements is in prediction of the optimum time to apply N fertilizer (Patriquin, 1978), with N fertilizer being applied during periods when exogenous NO_3^- could be metabolised rapidly and efficiently.

The problem with all the above proposed uses of NRA is that NRA is affected greatly by a wide variety of plant and environmental factors - as shown in detail by the work described here (Chapter 4) and in the work of others (Jung et al., 1980; Oosterhuis & Bate, 1983 and Sylvester-Bradley, 1984). The large effect of environmental factors on NRA may be largely due to the labile nature of the enzyme and its rapid turnover rate. Hence, NRA at the time of a test may not be exclusively related to the longer term N status of a crop.

It is hardly surprising, therefore, that many other workers have failed to find any significant correlations between NRA and crop yield or grain protein levels (Deckard & Busch, 1978), especially when assays in vitro are used (Dalling et al., 1975). NR assays in vitro are attractive to many workers, since very high NR levels are readily obtained, hence producing a very sensitive test. However, NR assays in vitro are likely to be especially unsuitable for determining correlations between NR and plant factors (e.g. yield, protein etc.) since they are usually performed under ideal (non-limiting) conditions of substrate and reductant availability - which is almost certainly not the case in growing plants.

Use of NR levels as a possible selection criterion in

breeding programmes for cultivars with potentially high yield or protein characteristics is widely proposed. (Austin et al., 1978). However, correlations found have, in general, not been very high (Beevers & Hageman, 1980) and have not been good enough to elicit significant interest from commercial plant breeders - the ultimate indicator of a successful test. The lack of high correlations is probably due to the lability of the NR enzyme and its susceptibility to massive variations due to fluctuations in environmental conditions.

These discouraging results have led some workers to reject the possibility of using NR measurements for prediction of yields or detection of promising cultivars (Naik et al., 1982 and Sylvester-Bradley, 1982), on the grounds that correlations found could not be repeated over a range of sites and from year to year. Likewise, the use of NRA levels as indicators of crop N status has been widely rejected (Bar-Akiva, 1970), once again due to the difficulty in determining reproducible "critical" enzyme levels - often as a consequence of the high sensitivity of NRA to variations in environmental factors.

For the reasons described above, it seems unlikely that "absolute" NRA measurements alone will ever be commercially useful as a predictive tool. However, the use of induced (NRA_i) and endogenous (NRA_e) enzyme activity measurements, and in particular the nitrate assimilation capacity ratio , NAC ($= {}^{NRA_i}/_{NRA_e}$) has revived hope for the useful development of this biochemical test (Bar-Akiva, 1970).

The difference between NRA_e and NRA_i values may be a good indicator of the extent of the nitrate assimilation capacity of a

crop, with a lack of difference between NRA_e and NRA_i values being an indication of N saturation (Bar-Akiva & Sternbaum, 1965). In terms of ratios of NRA_i to NRA_e (NAC ratios), a NAC ratio of close to 1 (i.e. NRA_i only slightly greater than NRA_e) indicates that the test crop is N sufficient, while successively larger NAC values (i.e. much induction of NRA during the NRA_i assay) indicate progressively increasing degrees of N starvation. These NAC values may therefore be of use as a measure of crop N status. The major problem remaining is then to correlate the crop's N status with the optimum rate and time of application of N fertiliser required.

As with NRA measurements, correlations of NAC with grain yield, N yield and % grain N of wheat have been fairly widely reported (Shaked et al., 1974; Verstraeten & Vlassak, 1981 and Verstraeten & Vlassak, 1982). Also, more success has been found in using NAC ratios, rather than NRA values alone, as predictors of plant N status (Shaked et al., 1974 and Taleisnik & Pachecho, 1980). The greater success of NAC as a predictive tool can be attributed mainly to the fact that NAC ratios have been found to be more stable to variations in plant and environmental conditions, unlike NRA values alone which fluctuate greatly (Bar-Akiva, 1970; Shaked et al., 1974; Taleisnik & Pachecho, 1980 and Davison & Stewart, 1983 and 1984).

In experiments in which NAC ratios have been related to plant N status, NAC values of ~ 1 have generally been found in crops fed large amounts of N (>1000kg N ha^{-1} for ryegrass) (Bar-Akiva, 1970). As lower amounts of N are used, increasing NAC values are found, up to a typical NAC value of ~ 10-20 when no N was supplied to the crop (Bar-Akiva, 1970). However, some workers have shown the test to

have an even wider range, with NAC values of up to 100 and more indicating increasingly severe N starvation (Verstraeten & Vlassak, 1982 and Verstraeten, 1983).

In addition, NAC values of ~ 1.5 have been found to correspond to optimal N nutrition in crops as different as ryegrass (Bar-Akiva, 1970), citrus (Bar-Akiva, 1970 and Shaked et al., 1974) and coffee (Taleisnik & Pachecho, 1980). This value of NAC might even be more widely applicable as a "critical" value for many crops for indicating optimal N status, with NAC values greater than 1.5 indicating that additional N fertilizer would be beneficial, and lower NAC values indicating that additional N fertilizer is not required.

It is likely that many of the workers who have rejected the use of NR as a predictive tool reached their conclusions prematurely, as a result of using unsuitable assay techniques (assays in vitro or non-optimal assays in vivo) in addition to the problems described previously. It was for this reason that it was considered essential to develop a sensitive, reproducible NR assay in vivo, rather than use a published technique, before attempting to use NRA as a predictive tool for determining crop N status or N fertilizer requirements.

The basic assay was developed and optimised as described in sections 3.3 to 3.4.9, and a wide range of pre-, during and post-incubation factors were studied, as reported in sections 3.4.10.2 to 3.4.10.16, with the work being summarised in section 3.4.11. The whole aim of the assay optimisation was to maximise NO_2^- accumulation (by enhancing NO_2^- production and minimising NO_2^- losses) during assays, since NO_2^- accumulation during NR assays in vivo is

taken as a measure of NRA. By doing this, the sensitivity and reproducibility of the basic assay technique was maximised.

The optimised assay technique developed has several important advantages over many earlier assay techniques, in that it is faster, highly sensitive and very repeatable. As many as 70 fresh leaf samples could be prepared and assayed within 3 hours by 2 people with the aid of automated NO_2^- analysis (by air segmented continuous flow or flow injection analysis). The technique is clearly not time consuming (unlike incubation methods of soil analysis or total N analyses of digested dry plant material), and many tests could therefore be readily performed routinely in a service laboratory.

As for sensitivity and precision, NRA_e values of as low as 0.03μmol NO_2^- g^{-1} fresh weight h^{-1} can be detected (e.g. section 4.5.2) and replicate analyses of subsamples drawn from the same bulked, chopped sample have a typical precision of better than $\pm$ 4%. The increased speed, sensitivity and precision gained is a clear justification for performing a detailed assay optimisation.

Now that a general method has been developed, such a process could (and should) readily be performed for each new crop species tested.

The routine use of the developed assay could also readily be adapted for field use. Rather than using a guillotine to chop samples for assays, leaf discs of optimal size could be cut quickly and accurately using a punch. A known number of these discs could then be assayed. This would increase rates at which samples could be handled - by eliminating the time needed to measure and cut leaf bundles and to weigh 500mg portions of leaf slices. Samples could then be incubated in Dewar flasks and NO_2^- could be determined by

comparison with a colour chart. This approach would be ideal for growers to use by themselves in the field and would probably not result in markedly increased errors in NRA determinations.

The only minor drawback of the NR assay is that the NED chemical used in the Griess Ilosvay/NO_2^- complex colour development is a powerful primary carcinogen. Hence coloured-up NR tests must be handled carefully and disposed of safely. An assumption made during the work is that optimal NR assay conditions are independent of conditions under which plants were grown. There was not sufficient time to investigate this assumption, but Jones and Sheard (1977) found it to hold for plants grown with different NO_3^- concentrations and at different light intensities.

The optimal NR assay in vivo developed was then used in the study of major plant factors which have been shown to affect absolute NRA of many plant species (variety, leaf position on the plant, leaf age, plant growth stage, part of leaf sampled), with the trends observed for barley being reported in sections 4.3.2 to 4.3.6 respectively. These marked influences of leaf position, leaf age and part of leaf assayed on NRA could easily confound results of studies of relationships between, for example, N fertilizer rates and NRA.

Many of the workers who have rejected the use of absolute NRA as a predictive tool for yield or N status may have done so as a result of the confounding effects of plant factors on NRA. However, the results reported in this work (section 4.3.5) suggest that the complicating effects on NRA due to leaf age, leaf position and part of leaf sampled could easily be avoided by assaying sub-samples drawn from bulked entire shoot material. The experiment (section 4.3.5)

was, however, only performed over a period of three weeks on young plants (between the 2nd and 5th leaf stages) and it is unlikely that NRA of entire shoot sub-samples would be invariant to leaf age and plant growth stage over the whole of a plants growth period.

After this study of effects of plant factors on NRA, the work moved on to investigate the effect of some of the major environmental factors which affect NRA. Factors investigated included light and dark (section 4.4), nutrient solution pH (section 4.6.5), root temperature (section 4.6.6) and shoot temperature (4.6.7).

The marked variations in NRA_e observed as a result of differences in light intensity under which plants were grown (section 4.4.2), and different times of sampling from the start of the photoperiod (section 4.4.3) would clearly confuse results of field experiments in which relationships between absolute NRA and N nutrition were being investigated. Sampling would therefore have to be done at the same time after the start of the photoperiod, and under similar conditions of prevailing (and probably also preceding) light intensity if the above problems were to be avoided. The measurement of light intensities (perhaps over several days) prior to sampling a crop for NRA determination, and being restricted to sampling at a certain time each day would be both time consuming and inconvenient.

Similarly, nutrient solution pH (section 4.6.5) and differential shoot and root growth temperatures (sections 4.6.6 and 4.6.7) affected NRA observed and would confuse results of field studies of the relationship between absolute NRA and crop N nutrition. All these results suggest that the study of relationships between NRA alone and field crop N nutrition would not be feasible due to the

variable effects of plant and environmental factors on NRA.

It might prove possible to allow for these effects of plant and environmental factors on NRA by producing equations by interpolation and extrapolation of the results obtained in this study. These equations could perhaps be used, along with details of the antecedent and prevailing plant and environmental conditions, to adjust measured NR activities to a "reference" value. By adjusting all NRA values to appear as they would under the same plant and environmental conditions, it might be possible to observe realistic relationships between NRA and crop N nutrition. However, the amount of computation and the accumulation of errors due to the modelling approximations would almost certainly make this approach unwieldy and unworkable. In addition, the results of this study were obtained under carefully controlled conditions, varying only one factor at a time, and it is almost certain that combinations of various factors, and variations from site to site and from season to season, would make the mathematical adjustment of NRA results to a set of standard conditions impossible.

For the reasons discussed above, it seems that the use of absolute NRA measurements alone would be of little use as a predictive tool for determining crop N status. Therefore, this project progressed to investigate the biochemical NR test in more detail. In the experiments described in section 4.6, both endogenous and induced NRA assays were performed and NAC ratios were calculated for each experimental treatment. NRA_e and NRA_i assays are readily performed using the optimal NR assay in vivo technique described earlier. They involve simply splitting a normal plant sample and assaying one half in a NO_3^--free medium (NRA_e) and the other in a

medium with added NO_3^- (NRA_i). Hence, the test remains simple and takes only slightly longer to perform.

The first promising attribute of NAC measurements over NRA values alone is illustrated in the results of sections 4.6.5 to 4.6.7. In these experiments, variations in nutrient solution pH and differential shoot and root temperatures were found to influence absolute NRA results. However, when ratios of NRA_i to NRA_e were calculated (i.e. NAC ratios), they were approximately constant, at a value of ~ 1.2. This NAC value is proposed to be a consequence of the plant's prevailing N status only. NAC values may therefore be specific indicators of crop N status and be invariant to influences of fluctuating nutrient solution pH and differential shoot and root temperatures. This would clearly be a great benefit in field studies of relationships between NAC and crop N nutrition.

A second useful point about NAC measurements is illustrated in the results of the experiment in which the form of N supplied to the plants was investigated (section 4.6.4). Form of N supplied (NH_4^+, NH_4NO_3 or NO_3^-) had a marked effect on absolute NRA measurements (more so than most of the variations due to plant and environmental factors even), but NAC values of plants supplied with mixed NH_4NO_3 or all NO_3^--N were only slightly different. Unfortunately, meaningful NAC values could not be calculated for plants fed only NH_4^+ (due to the very low NRA_e and NRA_i values recorded). However, it is unlikely that plants in the field would have NH_4^+ as their sole N source. Hence, it is suggested that NAC values of crops may be almost unaffected by the <u>form</u> of N supplied, and that the NAC values observed (~ 1.1-1.2 as found before) were a reflection of the plant's prevailing N status.

A large part of this work investigated the effect of NO_3^- nutrition on NRA under controlled conditions where other plant and environmental factors would not confuse results (sections 4.5.2-4.5.4 and section 4.6.3). The rapid induction of NRA over a few hours (section 4.5.3) by exogenously supplied NO_3^-, from zero in plants fed no NO_3^- (section 4.5.2), to a maximum within 48 hours after supply of NO_3^- (section 4.5.3) illustrates the sensitivity of NR to plant NO_3^- supply. In addition, the rapid decline in NRA over a period of 4 days after removal of NO_3^- nutrition (section 4.5.4) also shows the enzyme's rapid rate of turnover and fast response to changes in substrate concentration.

Similarly, the effect of the NO_3^- concentration supplied and maintained on barley NRA_e and NRA_i values was investigated in detail under carefully controlled environmental conditions (section 4.6.3). A clear relationship was observed, as shown in Figures 31 and 32, showing that NRA measurements could be correlated with the N status of these barley plants. A critical value of NRA could even be chosen, corresponding to the NO_3^- concentration required to produce optimum plant growth rates, yields or the desired high degree of N fertilizer utilisation as required.

However, it is almost certain that absolute NRA values so determined would be of no use for determining the N status of different field crops at various sites or from season to season, due to the massive variations in absolute NRA (which have been observed and documented in this project) as a result of many plant and environmental factors. Hence, the study continued to look more deeply at NAC ratios as a possible solution.

The NRA_e and NRA_i values obtained from samples of plants

grown over a range of NO_3^- feed concentrations (section 4.6.3) were ratioed, with the resulting trends being presented in Figures 33 and 34. There is a clear relationship between NAC ratio and NO_3^- concentration supplied. NAC ratios could therefore be of use as indicators of plant N status. This use of NAC ratios is also likely to be more suitable for field use than absolute NRA measurements alone, due to the demonstrated stability/tolerance of NAC ratios to variations in several major nutritional and environmental factors (e.g. form of N, section 4.6.4; nutrient solution pH, section 4.6.5 and differential shoot and root temperatures, sections 4.6.6 and 4.6.7).

The values of NAC observed for barley are of the same order as have been observed for crops as diverse as citrus (Bar-Akiva, 1970 and Shaked et al., 1974) and coffee (Taleisnik & Pachecho, 1980), with NAC values of greater than about 1.3 indicating sub-optimal N nutrition in barley. Unfortunately, the NAC test results for barley have a much narrower range (from about 1 when $\geq$ 32mg N l^{-1} were supplied to about 2.7 when only 2mg N l^{-1} were fed) than has been observed for other crops by several workers (who have observed NAC values of up to 100 and more in increasingly N-deficient plants) (Verstraeten & Vlassak, 1982 and Verstraeten, 1983). Due to this narrow range of the NAC ratio test, a corresponding problem arises with the sensitivity of the test being lower than would be desired. Small differences in crop NAC ratio could correspond to considerable differences in crop N status, and so even relatively small accumulations of errors in NAC determination (due to NRA_e and NRA_i assay methods, and any slight residual variations in NAC due to plant and environmental condition variations) could produce misleading

results. Hence, optimised assay in vivo techniques and careful testing are essential to obtain useful NAC results. Despite all the problems outlined above, the results of this initial study seem sufficiently encouraging to merit further investigation of NAC as a predictor of crop N fertilizer requirements and a small amount of time was made available to initiate a study of NAC in a field-grown barley crop.

This initial study of NAC of a field-grown, winter barley crop was described and the results documented, in section 4.5.6. The progressively increasing NAC ratios observed between December and March correspond to a decrease in the crop's N status and indicate a progressively increasing crop demand for N fertilizer. After N fertilizer top-dressing, the crop NAC ratio decreased rapidly to a value of 1.2-1.3, indicating that the crop's N demand had, to a large extent, been satisfied.

Results obtained in this brief field experiment seem encouraging enough to warrant a more extensive study in the future. Field NAC values observed here were of a similar magnitude to those observed under controlled environmental conditions (section 4.6.3). After the crop was given a typical (140kg N ha^{-1} in total) N fertilizer top dressing, the NAC ratio was observed to decrease to 1.2-1.3. This is close to the optimum NAC ratio observed under controlled environmental conditions, in this work, and in the work of others (Bar-Akiva, 1970; Shaked et al., 1974 and Taleisnik & Pachecho, 1980). These NAC values of 1.2-1.3 suggest that the N fertilizer top dressing rate used may have been close to the optimum. If an excessive amount of N fertilizer had been applied, a lower NAC value would probably have resulted (NAC close to 1). Similarly, if

too little fertilizer N had been added to the crop, higher NAC values than 1.3 would have been observed, indicating a sub-optimal N top-dressing for satisfying the crops N demand.

Although these initial results are promising, at present NAC ratios are really only of use as indicators of crop N status - and not yet as a predictive estimate of optimal N fertilizer top-dressing rates or times of N supply. Any future study to assess NAC as a commercially useful indicator must extend the work started here to find relationships between NAC and optimum rates and times of fertilizer N addition.

It is perhaps unrealistic to expect a single measurement - be it a soil, plant tissue or biochemical test - to be useful as a predictor of optimal fertilizer N rates and times of N top-dressing. It is almost certain that several such measurements would need to be made over a period of a few months (say January to May for a typical winter barley crop), to determine meaningful trends. It is also likely that even higher test frequencies would produce even more reliable results. In this respect, the NAC ratio test has the great advantage of being rapid and feasible for the grower to perform simply and regularly by himself (as described earlier). Hence, more frequent field testing could easily be done for NAC - unlike many soil and tissue analyses which require samples to be sent to a laboratory for ashing, digestion etc., and subsequent analysis.

If NAC ratios prove to be reliable indicators of crop N status, invariant to site and seasonal interfering factors, it is envisaged that NAC results gathered over the winter/spring period could be of use as predictors of both optimum rate and time of application of N fertilizer top-dressing. It is proposed that

growers would determine crop NAC ratios at regular intervals over the winter/spring period and progressively construct a graph of NAC ratio versus time. The graph could then be interpreted by the grower to determine the optimum time, and perhaps even the appropriate quantity of N fertilizer required by the crop. As winter progressed, the graph of NAC versus time would be expected to rise as the crop's N demand increased (i.e. N status decreasing as time passed). On the graph reaching a "critical" NAC value, the most appropriate time to apply N fertilizer top-dressing could potentially be determined.

By measuring the slope of the NAC versus time graph i.e. the rate of increase of NAC, an indication of how rapidly the crop was becoming N deficient would be obtained. This rate of increase in NAC could possibly even be related to the optimal quantity of N required to satisfy the crop's N demand - with crops showing rapid rates of NAC increase (i.e. metabolising N more rapidly) requiring larger quantities of N top-dressing. In addition, by following the trends in NAC as time progressed after top-dressing, further information could be obtained as regards the effectiveness of the top-dressing and the possible requirement for a second top-dressing.

After top-dressing, NAC would be expected to decrease rapidly (as seen in the preliminary field study carried out in this project), indicating that the crop's N demand had been satisfied to a certain extent. By following NAC trends after top-dressing, a measure of the extent to which the crop's N demand had been satisfied by the quantity of N top-dressing supplied could be obtained. The rate of increase in NAC, and the level to which NAC increased, after top-dressing could possibly even indicate the need for a second top-dressing - indicated by an increase in NAC to the critical level again.

This use of NAC as a predictor of rate and time of N fertilizer top-dressing is, of course, speculative at present. Much work is still required to accurately determine the critical NAC level for indicating the optimal time for N top-dressing to be applied. Likewise, many field trials will be required to determine the relationship between rate of NAC increase and rate of N top-dressing required. There was not enough time in the course of this project to perform these trials, it being thought more important to start at the beginning and optimise the assay and characterise NRA and NAC levels and variations in barley.

The results of this study seem encouraging enough to merit continuation of the work as outlined above. This work should provide a sound base for the further investigation of NAC as a predictive test of crop fertilizer N requirements, with potential commercial applications. It is possible that NAC alone may not be enough to predict accurately fertilizer N requirements, and may have to be used in conjunction with modelling of soil/plant factors to be of maximum use as a test for optimising N rates and times of addition.

6. CONCLUSIONS

The main conclusions reached and recommendations made as a result of this work are as follows:

6.1 Optimisation of the NR assay in vivo

a) Main assay conditions

Barley (var Igri) leaves or roots should be assayed for NRA_i in an incubation medium containing:

3%	(v/v)	propan-1-ol	(Section 3.4.2),
0.01%	(v/v)	Triton X-100	(Section 3.4.3),
0.02M		KNO_3	(Section 3.4.4),
and 0.1M		phosphate buffer	(Section 3.4.5).

KNO_3 is omitted from the medium if NRA_e is to be determined. Optimum incubation pH is 7.0 and 7.5 for leaf and root assays respectively (Section 3.4.6) and incubations are performed in darkness for 1 hour (Section 3.4.8) at a temperature of 32^oC (Section 3.4.7). Leaves are chopped into 5mm transverse slices (Section 3.4.9) and roots are chopped into approximately 10mm lengths.

b) Miscellaneous factors

i) Pre-incubation

- Neglectible levels of endogenous NO_2^- in the plants used for assays did not interfere with NR results (Section 3.4.10.2).
- Sample preparation times of up to one hour could be used without affecting NR results (Section 3.4.10.3).
- Vacuum infiltration is not recommended for routine use due to the extra time and equipment required (Section 3.4.10.4).
- Freeze/thaw treatment of samples must not be used, nor should plants to be used for NR assay be frozen (Section 3.4.10.5).

ii) During incubation

- All NO_2^- produced was due to enzymic activity, with no chemical NO_2^- production being observed (Section 3.4.10.6).
- NRA detected was solely due to plant material, with contributions from NR of contaminating bacteria being neglectable (Section 3.4.10.7).
- Waterlogging of tissues, produced by wetting effects of solvent and surfactant used, probably made conditions during assays anaerobic to the extent that N_2 flushing was unnecessary for maximising NO_2^- accumulation (Section 3.4.10.8).
- Shoot NR assays must be performed in darkness to minimise NO_2^- reduction by NiR (Section 3.4.10.9).
- Reduction of NO_2^- in darkness was not detected, but some losses of NO_2^- could not be discounted (Section 3.4.10.10).
- Incubation medium must be free from NH_4^+ to prevent reductions in NO_2^- production (Section 3.4.10.11).
- Reductant or carbohydrate supplies probably did not limit NO_3^- reduction in the plants used, since additions of sugars, NADH or L-malate did not increase NO_2^- production during NRA_e assays (Sections 3.4.10.12-3.4.10.14).

iii) Post-incubation

- Solutions removed from assays for NO_2^- determination can be stored (e.g. for subsequent automatic analysis), since leakage of active NR from plant tissues and further NO_2^- production in solution does not occur (Section 3.4.10.15).
- Post-incubation boiling of tissues in incubation medium is not recommended, since the extra time and equipment necessary is excessive in comparison with the slight increase in assay

sensitivity provided (Section 3.4.10.16).

6.2 Effects of plant factors and environmental conditions on NRA of barley

a) Plant factors

- True differences in NRA between barley varieties could not be unequivocally determined, since the incubation medium was optimised for variety Igri only (Section 4.3.2).
- It is recommended that entire shoots of plants be sampled, and that assay sub-samples be drawn from the bulked material (Section 4.3.5) for speed, and to avoid interfering effects of the observed dependence of NRA_e on part of leaf assayed (Section 4.3.6), leaf position on plant (Section 4.3.3) and leaf age (Section 4.3.4).

b) Light conditions

- Variations in light intensity have a relatively small effect on NRA because of the observed log-linear relationship between light intensity and shoot NRA (Section 4.4.2).
- Samples must be harvested at the same time each day to avoid interferences due to the marked diurnal variation noted in shoot NRA_e (Section 4.4.3).
- Shoot NRA declined exponentially to low values within 4 days after placing plants in darkness, due to lack of carbohydrate or reductant for NR, or to enzyme inactivation/inhibition (Section 4.4.4). However, NR activities returned to their previous high levels within 4 days after returning plants to the light (Section 4.4.5).

c) Nutrient pH

- The greater NO_3^- uptake by plants grown at lower pH may have increased the concentration of metabolically available, cytoplasmic NO_3^- and, hence, have induced the higher NR activities recorded in plants grown at lower pH (Section 4.6.5).

d) Growth temperature

- NRA was higher in roots of plants grown at lower root temperatures than in those grown at higher temperatures (Section 4.6.6). In addition, shoot NRA was higher in plants grown at lower shoot temperatures (Section 4.6.7). These effects were probably due to differential reduction in rates of NR synthesis/activation and degradation/inactivation at lower temperatures. The greater amounts of NO_3^- taken up by, and higher tissue NO_3^- contents of, plants grown at higher temperatures did not induce higher NRA, suggesting that greater amounts of NO_3^- may have been stored in vacuoles and, hence, have been unavailable as substrate for NR induction in these plants.

e) Nitrogen nutrition

- NO_3^- is essential for induction of NR, since no "background" NRA_e was detected in plants grown without NO_3^- (Section 4.5.2).
- NRA was induced rapidly (within a few hours) by NO_3^-, indicating fast NO_3^- uptake, transport and *de novo* synthesis of NR (Section 4.5.3). Induction continued for up to 48 hours, when maximum plateau levels of NRA were found, indicating

maximal induction under the prevailing conditions (Section 4.5.3).

- NO_3^- was essential for maintenance of high NRA and when NO_3^- nutrition ceased, NRA levels declined rapidly over a few days as the enzyme originally present was degraded or inactivated (Section 4.5.4).
- NH_4^+ exhibited a repressive or inhibitory effect on NO_3^- uptake and/or reduction by plants grown in nutrient solutions. Much more NH_4^+ than NO_3^- was taken up by plants when an NH_4NO_3 solution was supplied and maintained (Section 4.6.4). In addition, in an NH_4NO_3 depletion experiment (Section 4.5.5), NRA remained relatively low until most of the NH_4^+ was utilised. Thereafter, NRA increased to a maximum as inhibitory/repressive effects of NH_4^+ on NO_3^- uptake or reduction were removed.
- Shoot and root NRA_e and NRA_i increased asymptotically from near zero when no NO_3^- was supplied, to maximum, plateau levels when $\geq$ 16mg NO_3^--N l^{-1} were supplied in experiments in which various NO_3^- concentrations were maintained in nutrient solution (Section 4.6.3).

6.3 NAC

- In experiments in which environmental conditions were varied (e.g. nutrient solution pH (Section 4.6.5), root and shoot temperatures (Sections 4.6.6 and 4.6.7)), marked effects on shoot and/or root NRA_e and NRA_i values were observed. NAC, however, had the important advantage of being largely invariant to these different environmental conditions.
- NAC ratios also have the benefit of being almost invariant to

the form of N supplied to plants (mixed NH_4NO_3 or all NO_3^-), unlike NRA_e and NRA_i values which were greatly reduced in the presence of NH_4^+ (Section 4.6.4).

- Shoot NAC decreased asymptotically, from a maximum of 2.7 when 2mg NO_3^--N l^{-1} were supplied, to a minimum value of close to 1 when $\geq$ 16mg NO_3^--N l^{-1} were supplied in experiments in which a range of NO_3^- concentrations were supplied and maintained (Section 4.6.3). These NAC values are proposed to specifically reflect the plant's prevailing N status, unlike absolute NRA_e and NRA_i values which were shown to be influenced greatly by variations in plant and environmental factors (Chapter 4).
- In a preliminary field study, NAC of a winter barley crop increased progressively over the period December to March, indicating a progressively decreasing crop N status and an increasing need for N fertiliser top-dressing.
  After N top-dressing, NAC rapidly decreased (to a value of 1.2), indicating that the crop's N demand had, to a large extent, been satisfied, at least temporarily (Section 4.5.6).

6.4 Proposals for future work

Results of this initial study seem encouraging enough to warrant continuation of the project along the following lines:

- Investigations should be performed to determine if the demonstrated invariance of NAC to different environmental factors under controlled conditions applies in the field situation where several factors may vary simultaneously.
- The NR assay in vivo procedure should be developed into a test kit which the grower can use by himself in the field to

determine NAC ratios.

- Field studies should be performed to determine if NAC ratios can provide reproducible indications of crop N status from site to site and year to year.
- A "critical" NAC value for indicating the optimum time to apply N top-dressing to a field grown crop should be sought, as should the correlation (if any) between rates of increase in NAC and the optimum quantity of N top-dressing required.
- The possibility of using mathematical modelling in conjunction with NAC to produce an even more effective and widely applicable predictive tool should be investigated.

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